Hazem Al-Khawaja

Doxycycline inhibition of proteases and inflammation in abdominal aortic aneurysms

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Action is the foundational key to all success.

Pablo Picasso

Aan mijn familie

Colofon

The author changed his surname from Abdul Hussien to Al-Khawaja (medio 2010).

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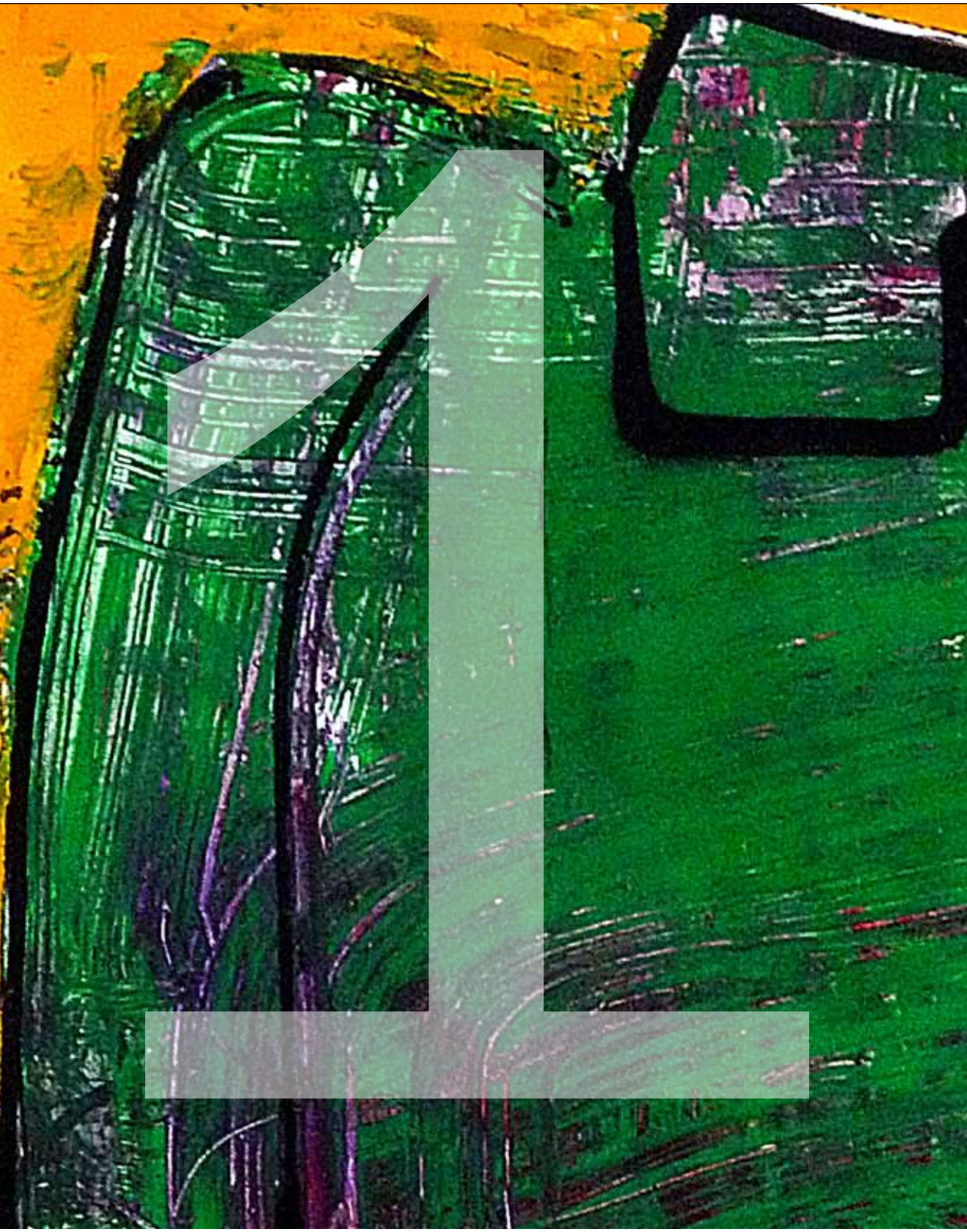


The Green Man, 2009

Introduction

An abdominal aortic aneurysm (AAA) is a progressive dilatation of the abdominal aorta that is most often located in the infrarenal aorta, i.e. between the level of the renal and the iliac arteries. In Western countries, the prevalence of AAA reaches approximately 10% among those over the age of 65.[1] Clinical risk factors that predispose individuals to have an aortic aneurysm include: smoking, advanced age, male gender, chronic obstructive pulmonary disease, hypertension, and a family history of aneurysmal disease.[2,3,4]

Atherosclerotic lesions are a common finding in pathological examinations of aneurysm walls. As such, the formation of aortic aneurysms has been attributed to a complication of atherosclerosis.[5] However, there is growing evidence suggesting that aneurysm formation is a multifactorial process that leads to defects in the structural components of the wall.[6] Progression of atherosclerosis may result in an occlusive disease, whereas aneurysm formation and progression represents a dilatation of the aortic wall. Atherosclerosis and aneurysmal disease have different risk factor profiles. Although elevated cholesterol level and hypertension are prominent risk factors for atherosclerosis, their role in AAA appears minimal.[7] Moreover, diabetes, an established risk factor for atherosclerosis, is not associated with AAA, and AAA diameter progression appears to be slower in patients with diabetes.[7] Further, smoking appears to be a stronger risk factor for AAA than for atherosclerosis.[8] Together, these observations suggest that AAA and atherosclerosis are different diseases and imply a more complex pathophysiology of aortic aneurysms than previously thought.

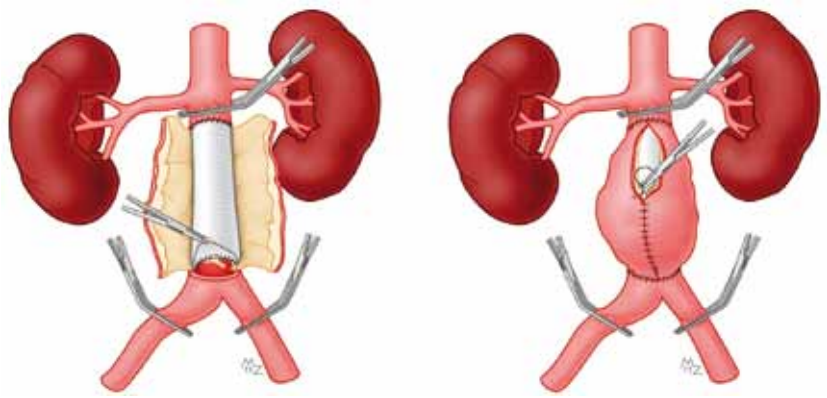
Typically, an aortic aneurysm enlarges slowly but without symptoms. At some point aneurysms may rupture, causing life-threatening bleeding. The risk of rupture is strongly determined by the diameter of the aneurysm. The risk of rupture of small aneurysms (i.e. a diameter of 5.5 cm or less) is very low (1–2% a year). The rupture risk, however, increases exponentially with an aneurysm diameter over 5.5 cm, and the estimated annual rupture rates of aneurysms over 7 cm exceed 15–20%.[9] Despite major improvements in medical care, the overall mortality rate for ruptured aneurysms remains as high as 60–80%.[1] Hence, current strategies for AAA are watchful waiting in small AAA, and elective repair of AAA over 5.5 cm.

Elective repair can either be done through an open abdominal operation or by an endovascular approach. The open abdominal approach, shown in Figure 1, is a major surgical procedure and has a significant complication risk. The mortality rate is approximately 3–5% and morbidity is significant.[10,11] Complications are commonly associated with patients with preexisting cardiovascular, pulmonary and renal conditions, the use of general anesthesia, and the duration of cross-clamp time of the aorta.[11]

The endovascular procedure is shown in Figure 2, and is generally considered a more elegant and less invasive approach. However, a large group of AAA patients with unfavorable anatomical AAA characteristics, such as severe aortic angulation and aneurysm neck morphology, cannot be repaired by endovascular techniques. Large clinical trials show that endovascular repair is associated with a significant lower operative mortality than

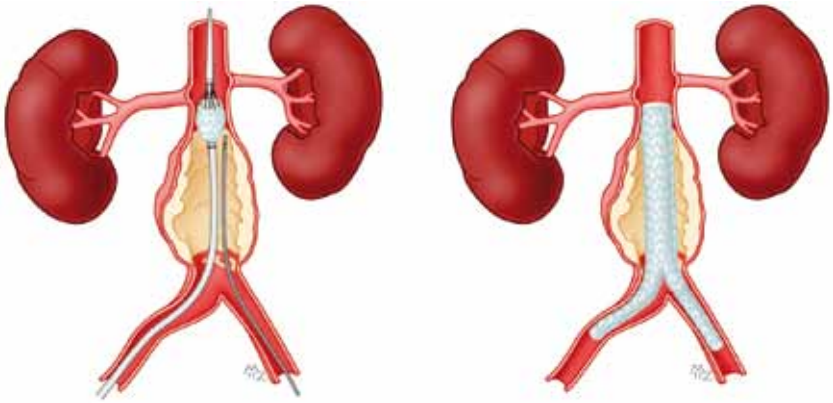
open repair. However, the long-term results in terms of total mortality or aneurysm-related mortality appear similar in both approaches.[12,13,14] Further, endovascular repair requires life-long follow-up because of the increased risk of graft-related complications, necessitating reinterventions.[15] Several studies indicate that endovascular repair is not cost-effective.[16,17]

FIGURE 1



In a conventional open aortic aneurysm repair, an abdominal incision is made to enter the peritoneal cavity. A prosthetic graft is used to replace the diseased segment of the aorta.

FIGURE 2



AAA illustrating endovascular stent graft placement. The procedure is done percutaneously. It usually involves two small incisions to expose the femoral arteries. A synthetic graft and stents are introduced through these arteries with guidewires and catheters until the graft is positioned correctly at the neck and normal iliac arteries. Removal of the sheath allows expansion of the stentgraft with barbs or other fixing devices to attach to the artery wall and hold the graft firmly in place, allowing blood to pass through it and remove pressure from the weakened aortic wall.

Randomized controlled trials comparing open repair versus conservative treatment dictate current operative management, for both open and endovascular treatment.[18,19] Current treatments are reserved for patients with aneurysms that have grown to more than 5.5 cm in diameter. Aneurysms smaller than 5.5 cm are not recommended for intervention since the risk does not outweigh the risk of future rupture of a small aneurysm.[18,19,20,21] The more widespread use of ultrasound screening and abdominal imaging has led to an increase in the number of AAAs referred for management.[22] Most abdominal aneurysms detected in screening programmes are smaller than 5.5 cm in diameter and operative treatment is not required. These patients are treated with a wait-and-see policy and they are usually followed by ultrasound. Remarkably, a large group of patients with a small aneurysm do never develop a large aneurysm that requires operative treatment due to the high incidence of cardiovascular disease and early mortality.[23]

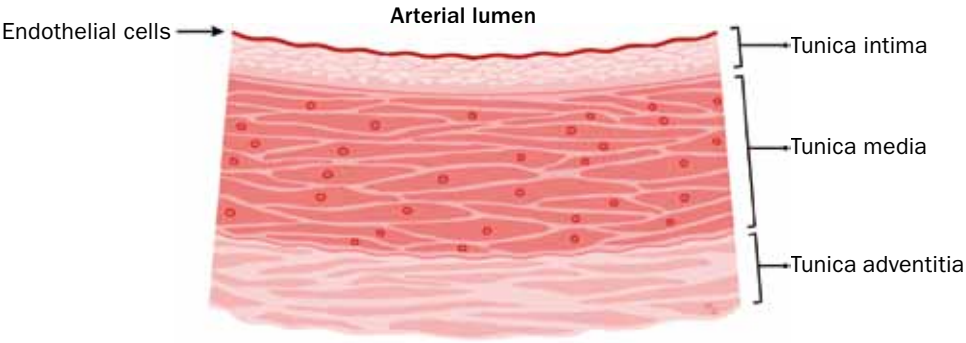
Current elective therapies have significant limitations: open repair is associated with a significant mortality and morbidity; whereas application of endovascular repair is limited by anatomical restrictions and concern over the long-term stability of the endograft. Moreover, the costs of treatment are high and are a major burden on the individual and society. Hence there is a need for a medical therapy that reduces the progression of small AAAs into large AAAs, which might decrease the risk of rupture and, consequently, the need for elective invasive treatment in patients with aneurysms. Moreover, medical therapy might be beneficial to reduce the need for repair and minimize the risk of rupture in patients with large AAAs unfit for aneurysm repair.[24] To develop such a medical therapy, a better understanding of the pathology of AAA is needed to develop pharmaceutical therapeutic strategies for aneurysm patients.

The pathophysiology of an AAA

The aorta is a so-called ‘elastic’ or conducting artery. The human aorta consists of three different layers; the tunica intima, the tunica media, and the tunica adventitia, as shown in Figure 3. The tunica intima is the innermost layer which consists of a monolayer of endothelial cells located on a basal lamina and a very thin layer of connective tissue.[25] The tunica intima acts as a semi-permeable barrier to the passage of molecules from the bloodstream into the arterial wall.[25] The tunica media is composed mainly of smooth muscle cells intermixed with elastin sheets, embedded in an extracellular matrix. It accounts for most of the elasticity of the arterial wall.[25] During systole, the elastic wall is stretched to accommodate the pressure volume of blood ejected from the heart, and subsequently the accumulated passive energy is released by a recoil action of the vessel, acting as a second subsidiary pump (known as the ‘Windkessel phenomenon’).[25,26,27] The outermost layer, the tunica adventitia, contains blood vessels required for nutrition of the outer layers of the vessel wall (vasa vasorum) which are embedded in collagen-rich connective tissue (mainly type I and III). The adventitia contributes significantly to the stability and strength of the arterial wall because of the dense network of collagen fibers which are neatly organized in a matrix or skeleton.[28] In diastole, or unstressed tissue, the collagen fibers are embedded in a wavy form, which causes the adventitia to be less stiff than the media in the stress-free

configuration; however, at significant levels of strain during systole, the collagen fibers reach their straightened maximum lengths and the adventitia becomes stiff. This mechanism prevents acute over-distension of the vessel wall, and in particular, of the smooth muscle cells in the media.[25]

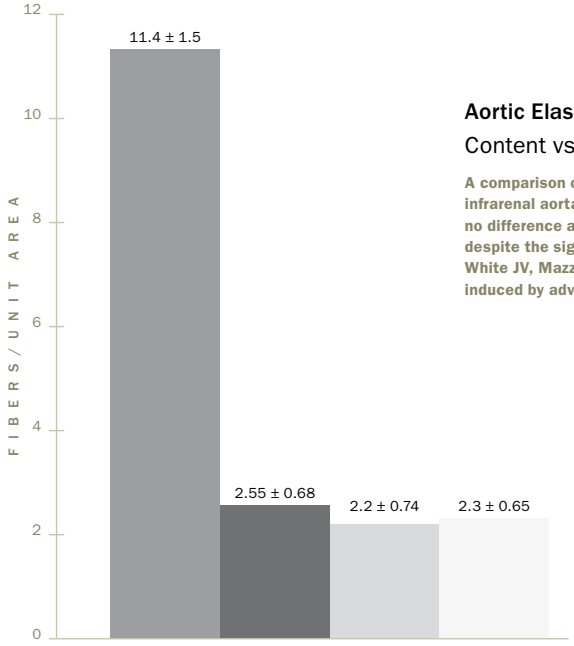
FIGURE 3



The different layers in an unaffected aorta.

The process of aneurysm formation and progression is associated with loss of the extracellular matrix components, elastin and collagen.[28] Although loss of elastin is the most prominent feature of AAA, it has been suggested that loss of collagen (fibrillar collagen type I and III) leads to the actual weakening of the arterial wall.[29] This has been supported by *in vitro* experiments showing that infusion of elastase in human aortic and iliac arteries only causes a slight vessel wall dilatation, whereas collagenase infusion leads to rapid dilatation and rupture.[30] Furthermore, the critical role of collagen in maintaining the integrity of the vessel wall is supported, as shown in Figure 4, by the observation that elastolysis is already complete in small aneurysms that are not prone to rupture. Hence progression and rupture of larger AAA represents degradation of the collagen matrix.[29]

FIGURE 4



Aortic Elastin

Content vs diameter

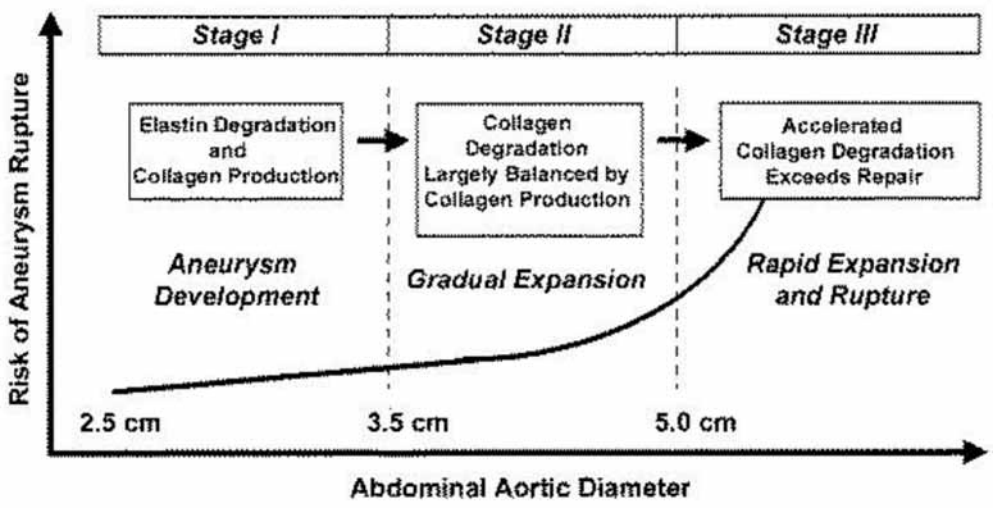
A comparison of the elastin concentration within normal human infrarenal aorta and small, moderate, and large aneurysms. There was no difference among the elastin concentrations of the aneurysm groups despite the significant differences in aneurysm diameter. Adapted from White JV, Mazzacco SL: Formation and growth of aortic aneurysms induced by adventitial elastolysis. Ann NY Acad Sci 1996;800:97–120.

Observations suggest that aneurysmal growth occurs through a gradually increasing imbalance between connective tissue degradation and repair. Thompson *et al*. proposed a model for aneurysm formation and growth, as shown in Figure 5.[31] Elastolysis is considered as the first step of aneurysm formation (AAAs <3.5 cm; stage I). Further progression of an aneurysm is caused by collagen degradation, which is replaced and repaired (stage II). Ultimately, the collagen degradation exceeds the collagen repair. This results in the fatal weakening of the vessel wall and subsequent rupture, which causes life-threatening hemorrhage (stage III). Preservation of this collagen network in the wall of the aorta may provide another way for reducing expansion of an aneurysm.[32]

Degradation of structural collagen involves two types of extracellular proteases: initial cleavage of the intact triple helix by collagenases, and subsequent degradation of the denatured or partially hydrolyzed forms of collagen into more soluble peptides by gelatinases.[33,34] Both activities have mainly been attributed to members of the matrix metalloproteinase (MMP) family and the cathepsins.[35,36]

The collagenases responsible for collagen degradation in the aneurysmal wall have not been fully identified. Reportedly, expression of MMP-gelatinase (MMP 2 and 9) as well as collagenase (MMP 1, 8 and 13) are increased in the wall of human aneurysms.[37,38] However, quantitative data on MMP expression and activity, and on their relationship to their inhibitors, are still absent. Furthermore, the involvement of other critical collagenases,

FIGURE 5



Stages of aneurysmal degeneration. Adapted from Thompson RW, Geraghty PJ, Lee JK. Abdominal aortic aneurysms: basic mechanisms and clinical implications. Curr Probl Surg. 2002;39:110–230.

such as the cysteine proteases cathepsin K, L, and S, has not been studied in the wall of human aneurysms.[39]

A role for MMPs in the progression and rupture of AAA has been shown in animal models. Inhibition of MMP activity (either by overexpression of tissue inhibitor of metalloproteinase (TIMP) or by doxycycline) provides protection against aneurysm formation.[40,41,42,43] This suggests that MMP inhibition may be an effective clinical strategy for the medical stabilization of AAA. Unfortunately, the use of specific MMP inhibitors such as batimastat and PG-116800 is limited by severe side effects on the musculoskeletal syndrome.[44]

Doxycycline

Doxycycline, a member of the tetracycline family of antibiotics, is well recognized for its ability to reduce MMP activity.[45] Coupled with its clinical availability, low cost and well-recognized safety profile, doxycycline seems to be an excellent candidate for evaluation of MMP inhibition in patients with an aneurysm. Doxycycline has been shown to suppress aneurysm formation in various animal models of AAA.[40,41,42,43] Moreover, doxycycline has been shown to attenuate or even stop aneurysm growth in two phase I/II studies: Mosorin *et al*. studied the effect of doxycycline in a small double-blind placebo-controlled study (n=32) and found that doxycycline significantly suppressed the expansion rate.[46] The authors reported that significant expansion (>5 mm in 18 months) occurred in five patients in the placebo group compared to only one patient in the doxycycline-treated group.[46] Baxter *et al*.

performed an open study in 36 patients with small abdominal aneurysm, and found evidence that doxycycline forestalled aneurysm growth during a six month follow-up.[47]

Doxycycline is a promising candidate for inhibiting aneurysm growth. Current studies attribute the beneficial effect of doxycycline to the inhibition of elastolytic MMPs (MMP 2 and 9). Yet, these proteases are unable to cleave intact structural collagens, which are critical for the stability of the vessel wall.[29] Arterial collagen degradation requires the action of specific collagenases that are able to cleave fibrillar collagen. So far, the effect of doxycycline on the possible inhibition of these collagenases has been not tested in human AAA.

Outline of the thesis

The aim of this thesis is to evaluate the effect of doxycycline on the proteolytic and inflammatory processes in abdominal aneurysms. This data is essential for the development of pharmaceutical strategies for the stabilization of an AAA. Such an approach could reduce the need for elective surgery and endovascular repair.

It has repeatedly been shown that AAA progression and rupture is related to the failure of collagen in the aortic wall. Yet the exact mechanism underlying this failure remains unknown. Furthermore, the precise mechanism of activation of collagenases and their inflammatory mediators that are responsible for the collagen turnover of AAA are unknown.

In this thesis we attempt to determine how collagen metabolism is balanced in aneurysmal diseases and contribute to the knowledge which collagenases and inflammatory mediators are involved in the destruction of the collagen network in AAA disease. Moreover, we evaluate some of the effects of doxycycline on the proteases and inflammatory mediators in AAA. Analyses showed that doxycycline inhibits specific MMPs and inflammatory pathways that are involved in the collagen balance and aneurysm growth. Together, these observations provide a rationale for a randomized clinical trial studying the effect of doxycycline on aneurysm growth.

Questions studied in this thesis:

- Which collagenases are involved in aneurysmal disease of the aorta? (**Chapter 2**)
- What is the effect of doxycycline on the collagenases in patients with an AAA? (**Chapter 3**)
- Which inflammatory pathways are involved in aneurysmal disease of the aorta? (**Chapter 4**)
- Is it possible to discriminate between primary and secondary upregulation of collagenases and inflammatory mediators in human AAA tissues? (**Chapter 5**)
- What is the effect of doxycycline on the inflammatory mediators in patients with AAA? (**Chapter 6**)
- Is it quality or quantity of the collagen that fails in aneurysm disease of the aorta? (**Chapter 7**)

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Happy birthday, 2009

Collagen degradation in the abdominal aneurysm: a conspiracy of matrix metalloproteinase and cysteine collagenases

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Abstract

Growth and rupture of abdominal aortic aneurysms (AAAs) result from increased collagen turnover. Collagen turnover critically depends on specific collagenases that cleave the triple helical region of fibrillar collagen. As yet, the collagenases responsible for collagen degradation in AAAs have not been identified. Increased type I collagen degradation products confirmed collagen turnover in AAAs (median values: <1, 43, and 108 ng/mg protein in control, growing, and ruptured AAAs, respectively). mRNA and protein analysis identified neutrophil collagenase [matrix metalloproteinase (MMP)-8] and cysteine collagenases cathepsin K, L, and S as the principle collagenases in growing and ruptured AAAs. Except for modestly increased MMP-14 mRNA levels, collagenase expression was similar in growing and ruptured AAAs (anteriorlateral wall). Evaluation of posttranslational regulation of protease activity showed a threefold increase in MMP-8, a fivefold increase in cathepsins K and L, and a 30-fold increase in cathepsin S activation in growing and ruptured AAAs. The presence of the osteoclastic proton pump indicated optimal conditions for extracellular cysteine protease activity. Protease inhibitor mRNA expression was similar in AAAs and controls, but AAA protein levels of cystatin C, the principle cysteine protease inhibitor, were profoundly reduced (>80%). We found indications that this secondary deficiency relates to cystatin C degradation by (neutrophil-derived) proteases.

Introduction

Abdominal aortic aneurysm (AAA) is a common pathology and a major cause of death because of rupture.[1,2] The hallmark pathology of AAA is a persistent proteolytic imbalance that results in excess matrix destruction and progressive weakening of the arterial wall. A number of matrix metalloproteinases (MMPs) (in particular the gelatinases MMP-2 and -9)[1,3] have been implicated as primary proteolytic culprits in the disease, but it is dubious whether these proteases are directly responsible for the weakening and ultimate failure of the aortic wall. Biomechanical studies invariably show that the mechanical stability of the arterial wall essentially relies on fibrillar collagens in media and adventitia.[4–6] These structural collagens are highly resistant toward proteolytic degradation, and the only mammalian proteases that have been shown to cleave the native triple helical region of fibrillar collagen are the classic collagenases of the MMP family[7] [i.e., MMP-1, -8, and -13 and the membrane type-1 MMP (MT-1 MMP or MMP-14[8])], as well as selected members of the cysteine protease family (ie, cathepsin K,[9,10] L,11 and possibly S[12]).

Several reports indicate expression of these collagenases in AAA on an individual basis, but comparative data regarding expression of the collagenases, and their possible relationship to rupture of the aneurysm,[13,14] are not available. Moreover, available studies[15] do not address the critical and complex posttranslational regulation of protease activity that involves controlled secretion of an inactive proenzyme, activation of the proenzyme, and rapid inhibition of protease activity by specific and nonspecific inhibitors.

To characterize collagenases involved in AAA growth and to test whether rupture is associated with increased collagenase expression, we used an integrated approach. We

first confirmed excess fibrillar collagen turnover in AAA and ruptured AAA wall samples through quantification of collagen degradation products and next established mRNA expression profiles of the MMP and cysteine collagenases by semi quantitative real-time polymerase chain reaction (RT-PCR). Because this approach does not provide information on the post-transcriptional regulation of protease activity, we quantified tissue expression of specific inhibitors of proteases activity and applied specific protease activity assays and Western blot analyses to address the post-translational regulation of protease activity.[16,17] Data from this study characterize members of the cysteine protease family, cathepsin K, L, and S, along with neutrophil collagenase (MMP-8), as the primary collagenases in AAA and ruptured AAA.

Materials and Methods

Patients

Tissue from the anterior-lateral aneurysm wall was obtained during elective surgery for asymptomatic AAA (-5.5 cm or larger, growing AAA) or during emergency surgery (ruptured AAA). Aortic patches removed along with the renal artery during kidney explantation from brain-dead, heart-beating, adult organ donors were used as controls. Samples were immediately halved. One half was snap-frozen in CO2-cooled isopentane or liquid N_2 and stored at -80°C for later analysis. The other half was fixed in formaldehyde (24 hours), decalcified (Kristensen's solution, 120 hours), and paraffin-embedded for histological analysis. Sample collection and handling was performed in accordance with the guidelines of the medical ethical committee of the Leiden University Medical Center, Leiden, The Netherlands.

RNA Isolation and Real-Time Competitive LightCycler PCR

RNA isolation and semi quantitative mRNA analysis using real-time competitive LightCycler PCR (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) were performed following protocols detailed by Lindeman and colleagues.[17] All mRNA data were normalized on basis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Tissue Homogenization

Aortic wall tissues were pulverized in liquid nitrogen and homogenized in lysis buffer [10 mmol/L Tris, pH 7.0, 0.1 mmol/L CaCl2, 0.1 mol/L NaCl, and 0.25% (v/v) Triton X-100]. This protocol releases both soluble as well as membrane-bound proteases. Samples were subsequently centrifuged at 13,000 rpm for 10 minutes at 4°C, snap-frozen, and stored at -80°C until use. Homogenates were normalized on the basis of their protein content (Pierce, Rockford, IL).

Collagen Degradation Assay

Collagen turn-over was assessed by the CTX assay for type I collagen degradation (Serum Cross laps; Nordic Biosciences, Milsbeek, The Netherlands). This assay is based on the detection of a neo-epitope that is released on cleavage of the GVG/L peptide bond in the C-terminal telopeptide of α2(I)-chain of mature type I collagen.

Specific Immunocapture MMP and Cathepsin Activity Assays

MMP-1, -8, -9, -13, and -14 (MT1-MMP) activity assays (Amersham Biosciences, Buckinghamshire, UK) were performed according to the suppliers' recommendations. In short, target proteases were captured by an immobilized specific antibody on microtiter plates, and the proteolytic activation of a modified proenzyme by the captured protease was used to quantify the protease activity.[17] MMP activity was quantified using recombinant MMP as standard. These assays have been shown to allow sensitive and specific assessment of active MMP as well as pro-MMP (on activation of latent MMP by a mercuric salt (*p*-aminophenylmercuric acetate) in *in vitro* systems. Conversely, assessment of active MMP in more complex samples such as tissue homogenates is generally hampered by rapid inactivation of active proteases because of the high levels of endogenous protease inhibitors that are present during initial sample preparation. Indeed, preliminary studies failed to indicate active MMPs in both aneurysmal and normal aortic wall homogenates; hence, only latent MMP activity (ie, on *p*-aminophenylmercuric acetate activation of the captured proenzyme) was assessed.

Cathepsin K activity was measured by a novel activity assay, based on the same principle as the MMP assays.[17] We developed a similar assay for assessment of cathepsin S activity; however, because of dissociation of the cathepsin S-cystatin C complex in the incubation steps required in the test, this assay measures both active cathepsin S as well as cathepsin S that was previously bound to cystatin C (cystatin C-complexed cathepsin S). Costar Stripwell plates were coated (2 hours, 37°C) with 1 μg/ml cathepsin S-specific monoclonal antibody (TNO-1503). This antibody does not cross-react with cathepsin K (<0.5%), L (0%), or V (<0.1%) and does not interfere with the enzyme activity. Purified cathepsin S (Calbiochem, Merck Biosciences, Darmstadt, Germany) or sample in binding buffer (20 mmol/L HEPES, 1 mmol/L ethylenediaminetetraacetic acid, and 0.1% Triton X-100, pH 6.5) were incubated for 16 hours at 4°C. Plates were subsequently washed four times with capture buffer (20 mmol/L HEPES, pH 6.5), and captured active cathepsin S was quantified through activation of a modified prourokinase variant (UKcatS) in detection buffer (20 mmol/L HEPES, 1 mmol/L ethylenediaminetetraacetic acid, and 0.1% Triton X-100, pH 8.5). Cathepsin S activation of the proenzyme was quantified using a chromogenic peptide substrate (S-2444). Cathepsin S activity was calculated from a standard curve using recombinant enzyme, and expressed in ng/ml. Thresholds for cathepsin K and S activity assays were 0.001 and 0.05 ng/ml, respectively.

Western Blot Analysis

Western blot analysis was used to quantify proteaseinhibitor complexes. Preliminary analysis showed that the primary antibodies used in the analysis allowed analysis of both pro- and active forms of the respective proteases and that the standard denaturing conditions required for Western blot analysis resulted in full dissociation of MMP-8-TIMP-1 and cathepsin-cystatin C complexes, thus indicating that the analysis allows assessment of MMP-8-TIMP and cathepsin K-, L-, and S-cystatin C complexes.

Western blot analyses for these proteases as well as for cystatin C and TIMP-1 were performed as described in Kleemann and colleagues,[18] using the following antibodies: anti-human cathepsin K (IM55L; Calbiochem, Breda, The Netherlands), anti-cathepsin L (AF952; R&D Systems, Abingdon, UK), anti-cathepsin S (sc-6505; Santa Cruz Biotechnology, Heerhugowaard, The Netherlands), anti MMP-8 (MAB3316; Chemicon, Chemicon Europe, Ltd., Chandlers Ford, UK), anti-cystatin C (sc-16989; Santa Cruz Biotechnology), and anti-TIMP-1 (Ab8229; Chemicon). All samples were normalized on the basis of total actin [anti-actin (sc-1615; Santa Cruz Biotechnology)] levels. All secondary antibodies were obtained from Santa Cruz Biotechnology. Immunoblots were visualized and quantified using Super Signal West dura extended duration substrate (Perbio Science, Etten-Leur, The Netherlands), LabWorks 4.6 software and the luminescent image workstation (UVP, Cambridge, UK).

Immunohistochemistry

Immunohistochemistry was performed using 4-μm deparaffinized, ethanol-dehydrated tissue sections. Sections were incubated overnight with a polyclonal antibody against MMP-8 (Medix Biochemica, Milsbeek, The Netherlands) or by a polyclonal antibody against the 100-kd transmembrane subunit of human osteoclast v-H+ATPase (a generous gift from Dr. M.A. Harrison, School of Biochemistry and Molecular Biology, University of Leeds, Leeds, UK).[16] Conjugated biotinylated antigoat or rabbit anti-IgG were used as secondary antibodies. Sections were stained with Nova Red (Vector Laboratories, Burlingame, CA) and counterstained with Mayer hematoxylin. Controls were performed by omitting the primary antibody.

In Vitro Cystatin C Degradation

Putative cystatin C degradation by MMP and serine proteases was evaluated in vitro. To that end, human cystatin C (5 ng/ml; Biovendor, Brno, Czech Republic) was incubated (24 hours, 37°C) with preactivated MMP-9 (0.3 ng/ml; Invitek, Leusden, The Netherlands), preactivated MMP-8 (0.3 ng/ml; Chemicon) or the serine protease neutrophil elastase (0.1 ng/ml; Calbiochem) in a 50 mmol/L Tris, pH 7.5, buffer containing 1.5 mmol/L NaCl, 1 μmol/L Zn+, 0.5 mmol/L Ca2+, 0.01% Brij, and 2 E/ml heparin, and remaining cystatin C was quantified by Western blotting (see above).

Statistical Analysis

mRNA expression (Ct values) was compared by Student's *t*-test or Wilcoxon-Mann-Whitney U-test. Results of activity assays and Western blots were analyzed by the Wilcoxon-Mann-Whitney U-test to compare the different groups. Putative correlations between cystatin C protein concentrations and cysteine protease mRNA and protein expression were analyzed by Pearsons' test. P values <0.05 were considered significant.

Results

Patients

Baseline clinical characteristics of the patients are provided in Table 1. Two of the AAA patients and two of the ruptured AAA patients had aneurysms elsewhere and one of the AAA patients had a family history of AAA. Because of national regulations, clinical data, other than sex and age, was not available for the controls; however, all donor organs were considered eligible for transplantation. The median age of the donors was 48 years (range, 27 to 76 years) and 45% were male.

TABLE 1

Patient Characteristics

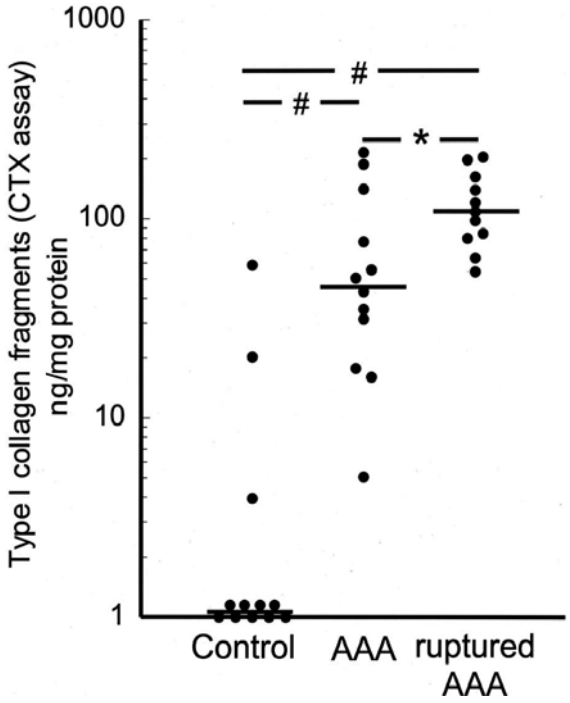
	AAA (n=17)	Ruptured AAA AAA (n=15)	P value
Age (years, mean±SD)	72.4 ± 6.2	72.5 ± 9.9	*P*=ns
Male/female	14/3	13/3	*P*=ns
AAA diameter (cm, mean±SD)	6.9 ± 1.3	7.7 ± 1.4	*P*=ns
Location AAA			
· Infrarenal	11	11	*P*=ns
· Juxtarenal	3	4	
· Suprarenal	3	0	
Smoking			
· Never	7	8	*P*=ns
· Stopped	7	5	
· Current	3	2	

ns, not significant.

Increased Collagen Turnover in AAA and Ruptured AAA

Sharply increased type I collagen carboxyterminal telopeptide fragments [CTX enzyme-linked immunosorbent assay (ELISA)] in AAA (AAA versus controls, $P < 0.001$; Figure 1) and a further increase in ruptured AAA (ruptured AAA versus AAA, $P < 0.02$) confirmed increased fibrillar collagen degradation in aneurysmal disease.

FIGURE 1

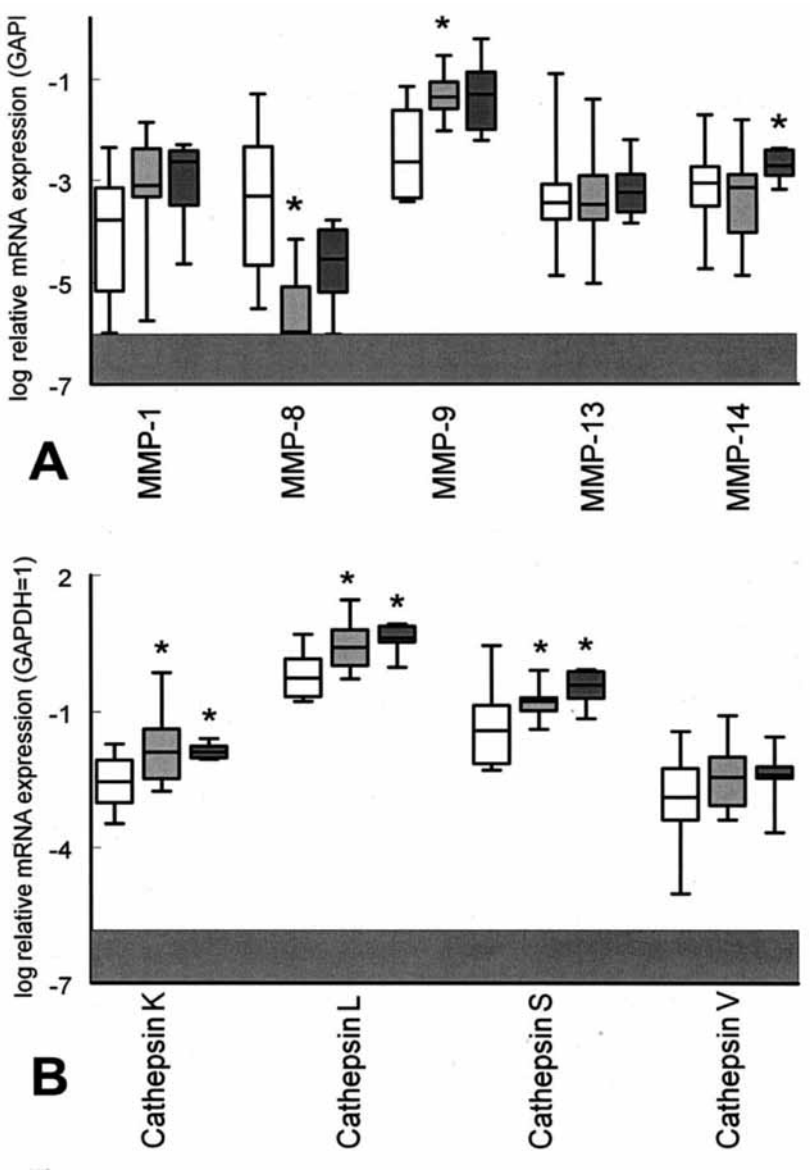


Collagen degradation (CTX assay for collagen type I degradation) in aortic wall samples of control aorta, growing AAAs, and ruptured aneurysms (ruptured AAAs). *P < 0.02; #P < 0.001.

mRNA Expression Profiles of MMP and Cathepsin Collagenases and Their Inhibitors

Normalized mRNA expression of MMP collagenases (MMP-1, -8, -13, -14), the gelatinase MMP-9 (an MMP gelatinase that is frequently associated with AAA), cysteine collagenases, and the endogenous inhibitors of protease activity is shown in Figure 2, A and B, and the normalized (GAPDH = 0) number of amplification cycles (Ct values) is provided in Table 2. Our findings confirm prominent expression of MMP-9 in AAA as well as in ruptured AAA ($P < 0.01$; Figure 2A, Table 2); however, with the sole exception of a modest increase in MMP-14 expression in ruptured AAA ($P < 0.05$), mRNA expression of MMP collagenases was similar and low in all three study groups (Figure 2A, Table 2). Expression of the cysteine collagenases cathepsin K, L, and S, on the other hand, was equally increased in both AAA and ruptured AAA ($P < 0.04$; Figure 2B, Table 2). Cathepsin V mRNA expression was similar and low in all groups. mRNA expression for the tissue inhibitors of MMP (TIMPs) as well as cystatin C, the cognate inhibitor of cysteine protease activity, is shown in Table 2. Expression of TIMP-1 and -3, the most prominently expressed TIMPs in the aortic wall, was similar and high in all study groups, whereas a moderate increase in TIMP-2 expression was observed in ruptured AAA ($P < 0.05$). Cystatin C expression was similar and high in all three study groups.

FIGURE 2



A: Relative mRNA expression (GAPDH=1) of MMP collagenases and the gelatinase MMP-9 in the infrarenal aorta of controls, growing AAAs, and ruptured aneurysms. *P< 0.05 versus controls.

B: Relative mRNA expression (GAPDH=1) of the cysteine collagenases in the infrarenal aorta of controls, growing AAAs, and ruptured AAAs. *P<0.05 versus controls.

TABLE 2

Normalized (GAPDH= 0) ΔCt Values of proteases and their inhibitors in Control Aorta, AAA, and Ruptured AAA.

	Control (n =11)	AAA (n=17)	Ruptured AAA (n=15)
MMP-1	14.2 [7.8 to 20.0]	9.7 [5.3 to 13.6]	9.4 [6.6 to 20]
MMP-8	11.9 [4.3 to 20]	15.9 [13.4 to 20.0]*	16.5 [12.6 to 20.0]*
MMP-9	7.7 (3.4)	4.0 (1.4)*	4.9 (1.8)*
MMP-13	11.5 (4.4)	10.1 (2.3)	9.9 (2.2)
MMP-14	10.1 (2.8)	10.7 (2.7)	9.4 (1.0)*
Cathepsin K	7.9 (2.3)	5.8 (2.3)*	6.4 (1.3)*
Cathepsin L	0.4 (1.9)	-1.6 (2.2)*	-2.3 (0.9)*
Cathepsin S	4.0 [-1.5 to 7.6]	2.2 [0.3 to 4.0]*	1.4 [-1.3 to 3.9]*
Cathepsin V	9.3 (3.5)	8.0 (2.4)	8.4 (2.1)
TIMP-1	-1.6 (1.6)	-2.7 (2.0)	-2.2 (0.9)
TIMP-2	5.7 (1.0)	4.2 (1.3)	4.9 (0.6)*
TIMP-3	1.5 (1.1)	1.6 (2.4)	2.0 (1.1)
Cystatin C	-3.1 (1.2)	-3.1 (1.1)	-3.1 (1.3)

Mean (SD) is in parentheses and median (range) is in brackets. Ct values represent the number of amplification cycles required before reaching a predefined threshold in the real-time PCR. All values were normalized on basis of duplex measurement of GAPDH expression. Normalized Ct values (ΔCt values) inversely relate to the mRNA expression (ie, a negative ΔCt value indicates expression exceeding GAPDH expression, whereas high Ct values indicate low mRNA expression). A ΔCt value of 20 reflects the detection limit of the assay (40 cycles). *P < 0.05 versus control.

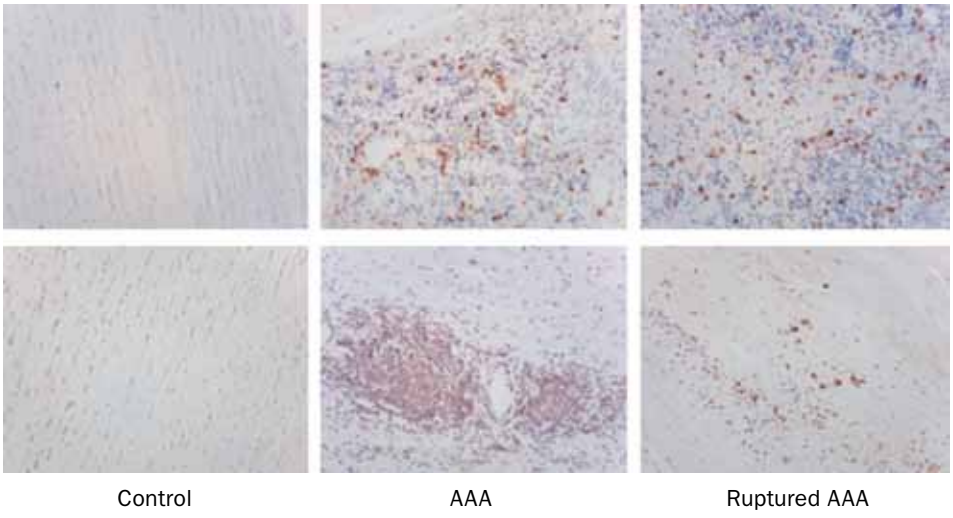
Protease Activity Assays and Western Blot Analysis

Posttranslational regulation of protease activity was evaluated by specific protein activity assays and Western blot analysis. Preliminary studies did not indicate direct MMP activity in the tissue homogenates (ie, activities below the detection limit of the respective assays (1.4, 5.0, 2.7, 8.1, and 0.2 ng/ml for MMP-1, -8, -9, -13, and -14, respectively); hence only latent (pro) forms (ie, on activation of captured latent MMPs) of the respective MMP collagenases were assessed. MMP-9 activities were included as positive control. Activation of captured latent MMP proteases revealed prominent MMP-8 [350 (161 to 622) ng eq/mg protein versus 275 (56 to 1361) ng eq/mg protein, median, (range); *P* = ns] and MMP-9 [84 (15 to 334) ng eq/mg protein versus 118 (68 to 1484) ng eq/mg protein in AAA and ruptured AAA, respectively, *P* = ns] activities, as well as marginal MMP-13 [37 (21 to 49) ng eq/mg and 34 (21 to 44) ng eq/mg protein, *P* = ns] proenzyme expression in AAA and ruptured AAA, respectively. Activities for MMP-1 remained below the detection threshold of the assay.

Immunohistochemical analysis of MMP-8 expression (Figure 3) showed that MMP-8 expression was primarily confined to infiltrating neutrophils, thus accounting for the apparent

discrepancy between minimal MMP-8 mRNA expression and abundant pro-MMP-8 activities. Unlike MMPs, cysteine protease are not readily activated by small molecular compounds; hence the cathepsin K activity assay only allows analysis of active cathepsin K. Analogous to assays for active MMP, the cathepsin K activity assay did not reveal net cathepsin K activity in AAA and ruptured AAA (detection threshold 0.001 ng/ml). We used a novel assay for the quantification of cathepsin S activity; however, do to the dissociation of cathepsin S-cystatin C complex in the assay, this assay measures both active cathepsin S as well as cystatin C-complexed cathepsin S. We found significant activities for cathepsin S in both AAA and ruptured AAA [37.3 (16.3 to 78.4) ng eq/mg protein and 49 (11.6 to 93.4) ng eq/mg protein (median ranges) in AAA and ruptured AAA, respectively; P = ns].

FIGURE 3



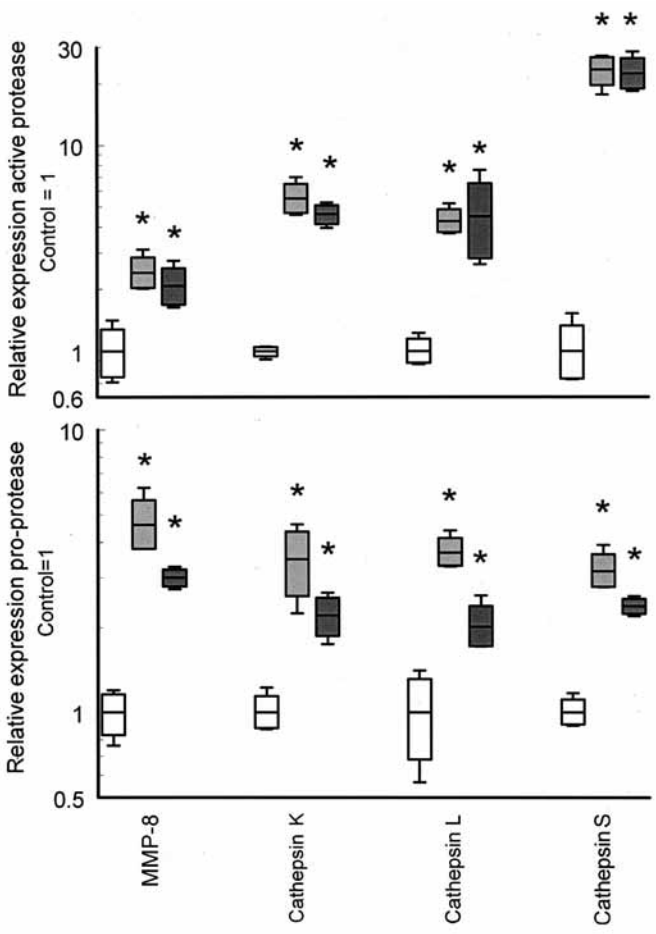
Immunohistochemical staining of MMP-8 (top) and the osteoclastic proton pump v-H+-ATPase (bottom) in normal control aorta, AAA, and ruptured AAA. MMP-8 is expressed in infiltrating neutrophils, whereas v-H+-ATPase is primarily expressed in monocytes/macrophages and to a lesser extent in smooth muscle cells.

Although detectable cathepsin S activities and absent MMP and cathepsin K activity may identify cathepsin S as the primary collagenase in AAA and ruptured AAA, these results mostly likely reflect the rapid inactivation of active proteases by excess endogenous protease inhibitors during the preparation of the tissue homogenates. It was reasoned that formation of protease-inhibitor complexes critically depends on preceding protease activation and that quantification of these complexes thus provides an indirect means of establishing preceding protease activation. We performed Western blot analysis for pro and active forms of MMP-8, cathepsin K, L, and S that indeed showed strongly increased activation of these proteases

in growing AAAs and ruptured AAAs (Figure 4). No differences were found between growing AAAs and ruptured AAAs.

Extracellular cathepsin K and L activity and stability critically depends on formation of an acidic pericellular microenvironment. In osteoclasts such an environment is created by the osteoclastic trans-membranous proton pump (v-H+-ATPase). Expression of this proton pump in cysteine protease-expressing mononuclear cells and smooth muscle cells in AAA and ruptured AAA (Figure 3) shows that conditions required for extracellular cathepsin K and L activities are present in aneurysmal disease.

FIGURE 4

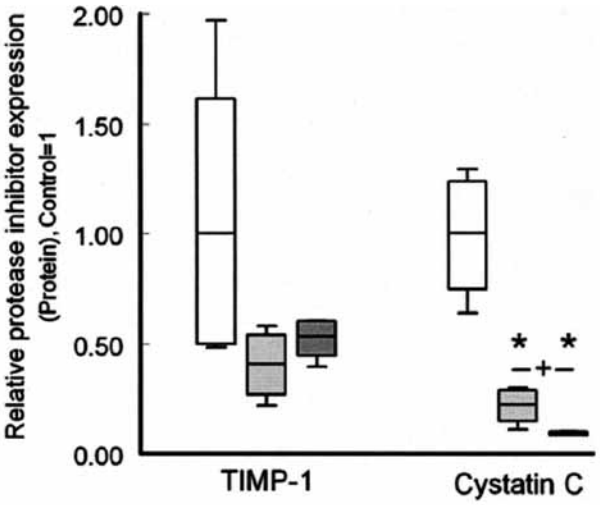


Increased expression of the activated forms of the collagenases MMP-8, cathepsin K, L (24- and 28-kd bands1), and S in growing AAAs (light gray boxes) and ruptured aneurysms (dark gray boxes) compared with control aorta (white boxes). *P<0.05 versus controls.

TIMP-1 and Cystatin C Protein Expression

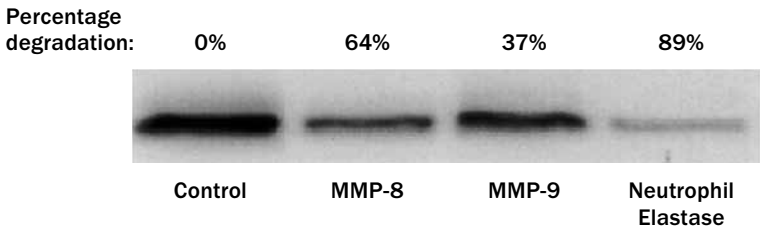
Protein expression of TIMP-1, the principal TIMP in the arterial wall, as well as expression of cystatin C was evaluated by Western blot analysis. Data in Figure 5 suggest a trend toward lower TIMP-1 protein expression in AAA and ruptured AAA; however, this difference did not reach significance ($P < 0.1$). Cystatin C protein levels were significantly reduced in growing AAA ($P < 0.05$) and further ruptured AAA ($P < 0.05$ versus growing AAA). Decreased cystatin C protein levels along with abundant cystatin C mRNA expression in AAA suggests that the decreased cystatin C levels in AAA are secondary and may relate to increased cystatin C consumption as result of increased cysteine protease activity or alternatively may reflect increased cystatin C catabolism. We found no indication for an association between reduced cystatin C protein levels and cysteine collagenase mRNA or protein expression (data not shown). However, the observed inverse relationship between active MMP-8 and cystatin C protein levels ($r = -0.78$, $P < 0.05$) in growing AAA suggests that cystatin C deficiency is secondary and may result from proteolytic degradation by MMP-8 or other neutrophil-derived proteases. Indeed, *in vitro* experiments showed that cystatin C is degraded by various neutrophil-derived proteases such as MMP-8 and the serine protease neutrophil elastase, and to a lesser extend by MMP-9 (Figure 6).

FIGURE 5



Relative protein expression of the principal inhibitor of MMP activity (TIMP-1) and of cystatin C, the principal inhibitor of cysteine protease activity in control infrarenal aorta (white boxes), growing AAAs (light gray boxes), and ruptured AAAs (dark gray boxes). *P<0.05 versus controls;+P<0.05 AAA versus ruptured AAAs.

FIGURE 5



Cystatin C degradation by neutrophil proteases MMP-8, MMP-9, and neutrophil elastase *in vitro*. Cystatin C and respective proteases were incubated for 24 hours in a 100:1 mol/mol ratio.

Discussion

Biomechanical[4–6] and clinical studies[19] demonstrate that the mechanical strength of the vascular wall relies on the structural collagen network in the media and adventitia. Increased collagen turnover that is not adequately matched by collagen deposition is held responsible for the growth and ultimate rupture of AAA.[1] However, the proteases responsible for the increased collagen turnover have not been identified. Load-bearing collagens within the arterial wall are predominantly type I/III fibrillar collagens that are highly resistant toward proteolysis. Degradation of these collagens critically depends on the action of specific collagenases that are able to destabilize the triple helix of native fibrillar collagen.[20] Destabilized collagen helices can than be further degraded by less specific proteases such as the gelatinases MMP-2 and -9, and the stromelysins MMP-3 and -10. Hence, degradation of collagen matrix in arterial wall primarily dependent on the initial action of specific collagenases (ie, the classic MMP collagenases as well as selected members of the cysteine protease family).

Several reports indicate expression of MMP as well as cysteine collagenases in AAA on an individual basis,[21–26] but these studies[15] are not quantitative and do not address the important posttranslational regulation of protease activity, which involves controlled activation of the inactive proenzyme and subsequent inactivation by specific and nonspecific endogenous inhibitors.[16,27] Moreover, the possible involvement of increased collagenase activity[13,14] as the underlying cause of rupture has not been addressed in detail.

To confirm increased collagen turnover in AAA and to identify candidate collagenases involved in AAA growth and possible rupture, we used an integrated approach that involved evaluation of collagen degradation, expression of all MMP and cysteine collagen-degrading enzymes, and the posttranslational regulation of protease activity. Putative increases in aortic wall collagen turnover[1] were evaluated by the Serum Crosslaps ELISA. This ELISA specifically detects C-telopeptide fragments that are released on proteolytic cleavage of native type I fibrillar collagen. Sharp increases in C-telopeptide fragments in AAA wall samples, and an even further increase in wall samples of ruptured AAA, confirms increased fibrillar collagen degradation in AAA and corroborates earlier observations of increased collagen degradation in ruptured AAA.[13,14]

To identify collagenases responsible for the excess collagen degradation, we first explored mRNA expression of the classic collagenases (namely the MMP collagenases, MMP-1, -8, -13, and -14) by semi quantitative real-time PCR. We included expression of MMP-9, a gelatinase that is prominently expressed in AAA, as the positive control. Findings from the mRNA analysis confirmed prominent expression of MMP-9 in AAA3 and ruptured AAA. With the exception of a modest increase in MMP-14 expression in the ruptured AAA, analysis did not indicate increased MMP collagenase mRNA expression in growing AAA or ruptured AAA. We used specific immunocapture-protease activity assays to validate the MMP mRNA data. These activity assays have been shown to allow quantification of active proteases[16] and, after *in vitro* activation of the latent MMPs, assessment of the pool of pro-MMP.[16] Direct assessment of MMP collagenases (ie, active enzymes) did not reveal detectable protease activity in the tissue homogenates (all activities were below the detection threshold of the assay). Although this finding may indicate that all collagenases present are in their inactive, latent form, it most likely reflects a technical limitation when assessing protease activity in complex biological samples such as tissue homogenates. Under such conditions, high levels of endogenous inhibitor will rapidly inactivate any active protease present, thus resulting in the absence of detectable protease activity.

Findings for the latent (pro) MMPs (ie, on *in vitro* activation of the latent proteases) primarily paralleled findings from mRNA analysis and indicated significant expression of proMMP-9 but only minimal expression of the collagenases pro-MMP-1 and -13. Indicating that the absolute expression of MMP-1 and -13 in AAA[23–25] is low, suggesting that their contribution to collagen degradation in growing and ruptured AAA is limited. Prominent pro-MMP-8 activities sharply contrast with minimal MMP-8 mRNA expression, and our activity data actually put MMP-8 on par with MMP-9 as the most prominently expressed MMP in AAA. Neutrophil MMP-8 is a stored secondary granule protein that is transiently expressed during the late myeloid maturation pathway of neutrophils[28,29] Immunohistochemical analysis confirmed MMP-8 abundance in growing and ruptured AAA and showed that MMP-8 is predominantly expressed in infiltrating neutrophils, thus accounting for the apparent discrepancy between MMP-8 mRNA and protein expression.

The activity assays did not indicate net MMP-8 activity. Failure to detect any appreciable MMP-8 activity most likely relates to inactivation of active proteases by excess endogenous protease inhibitor during preparation of tissue homogenates. This notion is supported by our observation of increased MMP-8 inhibitor complexes (Western blot analysis) in growing and ruptured AAA. Formation of these complexes critically depends on protease activation, and assessment of protease-inhibitor complexes thus provides a means of establishing preceding protease activation. We validated Western blot analysis as a means of quantifying protease inhibitor complexes and found abundant expression of the active 28-kd MMP-8 form in growing and ruptured AAA, thus showing that MMP-8 activation had occurred in AAA and ruptured AAA.

Although the MMP-collagenases are referred to as the classic collagenases, it is now apparent that selected members of the cysteine family of proteases are involved in remodeling of the collagen matrix as well.[30] Extracellular activities of cathepsin K and L have been recognized as critical factors in bone turnover[31] and endothelial stem cell trafficking,[32] and evidence from animal studies identifies cathepsin K and S as critical factors in remodeling of the atherosclerotic plaque.[33] Sharply increased C-telopeptide fragments (CrossLaps ELISA) in aortic wall samples of AAA and an even further increase in ruptured AAA show that the cysteine proteases are also involved in collagen degradation in growing and ruptured AAA.[34] We analyzed mRNA expression of the cysteine proteases cathepsin K, L, S, and V[35] as well as expression of cystatin C, the cognate endogenous inhibitor of extracellular cysteine protease activity. In contrast to the data for the MMP-collagenases, this analysis indicated clear increases in mRNA expression of cathepsin K, L, and S in both AAA as well as ruptured AAA. Again, no apparent differences were found between growing and ruptured AAA. Activation[36,37] and stability of cysteine proteases cathepsin K and L critically relies on an acidic pericellular environment.[38–40] In osteoclasts such a microenvironment is created by a transmembraneous proton pump v-H+-ATPase.[41] We performed immunohistochemical staining for this osteoclastic v-H+-ATPase[17] and found abundant expression of this proton pump in infiltrating mononuclear cells and to a lesser extend in the vascular smooth muscle cells in growing and ruptured AAA, indicating that the optimal conditions required for pericellular cysteine protease activity may indeed exist in aneurysmal disease.

We used novel specific activity assays based on the same principle as the MMP activity assays to evaluate cathepsin K[17] and S activities in AAA and ruptured AAA. Akin to the MMP activity assays, the cathepsin K activity assay did not indicate net cathepsin K activity, but we did observe significant cathepsin S activity in growing AAAs and ruptured AAAs in the cathepsin S activity assay. Abundance of activated cathepsin K by Western blot analysis shows that cathepsin K activation occurs in AAA and indicates that failure to detect active cathepsin K in the activity assay presumably reflects inactivation of active cathepsin K by the endogenous inhibitors during preparation of the tissue homogenates. Abundant cathepsin S activities in the novel cathepsin S assay may identify cathepsin S as the principal collagenase in AAA and ruptured AAA; however, we found indications that observed cathepsin S activities relate to dissociation of the cathepsin S-cystatin C complex during the washing steps required in the cathepsin S activity assay, an effect that is not seen in MMP and cathepsin K activity assays.

Reported deficiencies in cystatin C, the principle inhibitor of extracellular cysteine protease activity[42,43] in AAA may amplify the role of the cysteine proteases. It was postulated that these deficiencies occur at the transcriptional level and relate to transforming growth factor-deficiency.[42] However, our data point to a different mechanism. Reduced protein levels, albeit similar cystatin C mRNA expression, along with the inverse relationship between tissue MMP-8 and cystatin C levels and our *in vitro* data showing that cystatin C is degraded by various neutrophil-derived proteases such as neutrophil elastase and MMP-8, suggest that cystatin C deficiency in AAA is secondary and may relate to cystatin C degradation by

neutrophil-derived proteases. Such a mechanism is not known for cystatin C, but a similar gain of function mechanism[44] has previously been described for the serpin α1-proteinase inhibitor.[45] Eliason and colleagues[46] recently showed that neutrophil depletion inhibits aneurysm formation in the elastase model of aneurysm formation but also observed that, although neutrophils are critical for the process of aneurysm formation in this model, their contribution is independent of MMP-8 (as well as of MMP-2 and -9). Neutrophil-mediated cystatin C degradation may well explain the putative prominent role of neutrophils in the process of aneurysm formation.

In conclusion, our results confirm excess collagen degradation in AAA and ruptured AAA and identify MMP-8 and the cysteine proteases cathepsin K, L, and S that are expressed along with the osteoclastic proton pump v-H+-ATPase as the principle collagenolytic culprits in AAA. Our findings confirm and extend findings from Wilson and colleagues[47] but do not indicate increased MMP or cysteine collagenase expression in the anterior aneurysmal wall as the cause of rupture,[47,48] yet we cannot exclude that local increases in cysteine collagenase activities at the site of rupture contribute to rupture of the aneurysm. Reduced cystatin C protein expression along with increased collagen degradation products in the anterior aneurysmal wall of ruptured aneurysms points to an alternative mechanism and suggests that protease inhibitor deficiency rather than increased protease expression may contribute to AAA rupture. Pharmaceutical inhibition of cysteine protease activity[49] and/or manipulation of neutrophil activation[50] may provide a pharmaceutical means of stabilizing AAA.[51]

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Doxycycline therapy for abdominal aneurysm: improved proteolytic balance through reduced neutrophil content

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Abstract

Background: Matrix metalloproteinase-9 (MMP-9) is thought to play a central role in abdominal aortic aneurysm (AAA) initiation. Doxycycline, a tetracycline analogue, has direct MMP-9-inhibiting properties in vitro, and it effectively suppresses AAA development in rodents. Observed inhibition of AAA progression, and contradictory findings in human studies evaluating the effect of doxycycline therapy on aortic wall MMP-9, suggest that the effects of doxycycline extend beyond MMP-9 inhibition and that the effect may be dose-dependent.

Methods: This clinical trial evaluated the effect of 2 weeks of low- (50 mg/d), medium- (100 mg/d), or high-dose (300 mg/d) doxycycline vs no medication in four groups of 15 patients undergoing elective AAA repair. The effect of doxycycline treatment on MMP and cysteine proteases, and their respective inhibitors, was evaluated by quantitative polymerase chain reaction, Western blot analysis, immunocapture protease activity assays, and immunohistochemistry.

Results: Doxycycline was well tolerated and no participants dropped out. Doxycycline treatment reduced aortic wall MMP-3 and MMP-25 messenger RNA expression ($P < 0.045$ and $P < 0.014$, respectively), selectively suppressed neutrophil collagenase and gelatinase (MMP-8 and MMP-9) protein levels ($P < 0.013$ and <0.004, respectively), and increased protein levels of the protease inhibitors tissue inhibitor of metalloproteinase 1 and cystatin C ($P < 0.029$). As for the apparent selective effect on neutrophil-associated proteases, we sought for a reducing effect on aortic wall neutrophil content that was indeed confirmed by immunohistochemical analysis that revealed a 75% reduction in aneurysm wall neutrophil content ($P < .001$).

Conclusions: Independent of its dose, short-term preoperative doxycycline therapy improves the proteolytic balance in AAA, presumably through an effect on aortic wall neutrophil content. This study provides a rationale for doxycycline treatment in patients with an AAA as well as in other (vascular) conditions involving neutrophil influx such as Kawasaki disease and Behçet disease.

Clinical Relevance: The concept of pharmaceutical stabilization of abdominal aneurysms is promising. Doxycycline, a tetracycline analogue, is considered a lead candidate, but its mode of action is still unclear. This clinical trial showed that doxycycline treatment, through a profound effect on the number aortic wall neutrophils, has a pronounced but selective effect on the proteolytic balance in the abdominal aneurysm, as indicated by reduced matrix metalloproteinase (MMP)-8 and -9 levels and concentrations of tissue inhibitor of metalloproteinase-1 and cystatin C. The observation that doxycycline has a selective effect on neutrophil-derived proteases is remarkable and novel, and suggests that doxycycline may also be effective in other vascular conditions involving neutrophils, such as Kawasaki disease and Behçet disease, and nonvascular conditions such as chronic obstructive pulmonary disease.

Introduction

An aneurysm of the abdominal aorta (AAA) is a common pathology and a major cause of death due to rupture.[1] Risk of rupture is negligible in small AAAs, but increases exponentially in AAAs with a diameter of ≥55 mm.[1] Hence, current approaches toward AAAs are surveillance of small AAAs and preventive surgical repair of larger AAAs (i.e., ≥55 mm).[2] Unfortunately, conventional transabdominal (open) repair is associated with considerable morbidity and mortality.[3] Although short-term results of endovascular repair appear more favorable, rates of mid- and long term mortality are similar to those of conventional repair.[4,5] Endovascular repair, moreover, requires life-long follow up, and the number of reinterventions after endovascular repair is high (approximately 9%), although most are minor.[6] As a consequence, the effectiveness of endovascular AAA repair is now being challenged.[7,8]

Pharmaceutical therapy inhibiting aneurysmal growth, and thus reducing the need for invasive treatment, could have major advantages for patients as well as socioeconomic benefits.[9] Excess matrix degradation that is not balanced by matrix deposition is considered pivotal to aneurismal growth.[1] This led to the proposal that pharmaceutical intervention that would reduce protease activity, in particular, matrix metalloproteinase-9 (MMP-9), could restore the balance between matrix degradation and deposition and thus reduce aneurysmal growth.[10,11]

Members of the tetracycline family of antibiotics have been recognized as inhibitors of MMP expression and activity. It was thus proposed that doxycycline, a tetracycline analogue, may reduce excess MMP-9 activity in AAA and constitute a pharmaceutical means of reducing aneurismal growth.[10,11] Indeed, doxycycline has been shown to inhibit aneurysm formation in various animal models of AAA.[10-13] Moreover, results from two preliminary phase I/II studies in patients under surveillance for AAA suggest that long-term doxycycline treatment may also attenuate or even forestall aneurysm growth.[14,15]

Despite these promising findings, a number of questions, in particular the mode of action of the drug,[16] remain to be answered. The rationale behind doxycycline therapy was to prevent aneurysmal growth through inhibition of elastolysis mediated by MMP-9.[10,17] Yet, it is now recognized that loss of elastin is a very early event in AAA formation and that aneurysmal growth and ultimate rupture essentially depend on loss of structural collagens.[1,18] However, MMP-9 is a gelatinase that can only degrade collagen after initial cleavage by specific collagenases.[18] Observed inhibition of aneurysmal growth in animal models and human studies therefore suggests that apart from its effects on MMP-9, doxycycline therapy also influences the collagenases.

A recent study[19] evaluated the effect of preoperative doxycycline treatment on messenger RNA (mRNA) expression of the collagenases MMP-1, MMP-13, and MMP-14, but failed to observe an effect on these proteases. The effects on the primary collagenases in AAA (MMP-8 and the cysteine proteases cathepsins K, L, or S)[18] were not evaluated.

Another issue that has not been addressed is the contradictory results of human studies evaluating the effects of doxycycline on MMP-9 expression and activity. Although the initial human study showed that preoperative doxycycline reduced MMP-9 mRNA expression and activity,[17] a later more elaborate study failed to observe such an effect.[19] The basis for these divergent findings is unclear, but they may reflect increased MMP-9 expression as result of an immunostimulatory effect on monocytes as seen at higher doses of tetracyclines[20]; indicating that the contrasting findings in human studies may reflect a dose–response relationship.

To evaluate the effects of doxycycline therapy on the MMP and cysteine proteases,[18] their respective inhibitors,[18,21] and to test whether the apparent contradictory findings of human studies on MMP-9 relate to dose dependency, we evaluated the effect of low (50 mg/d) regular (100 mg/d) and high (300 mg/d) doses of doxycycline on the members of the MMP family of proteases and the cysteine collagenases cathepsin K, L, and S. Results from this study show that, irrespective of dose, 2 weeks of preoperative doxycycline treatment improves the proteolytic balance through reduction of the neutrophilassociated proteases MMP-8, MMP-9 and MMP-25, and an increase of the protease inhibitors tissue inhibitor of metalloproteinase 1 (TIMP1) and cystatin C. Further evaluation of this phenomenon showed that doxycycline therapy profoundly reduced the number of infiltrating neutrophils in the aneurysmal wall.

Materials and Methods

Patients

The study was performed on intention-to treat basis. The study included 60 patients scheduled for elective, open aneurysm repair and excluded patients with chronic inflammatory disease or inflammatory aortic aneurysms. Decisions for open repair were based on anatomic (e.g., neck, elongation) and patient characteristics (age), and on patient preferences. Patients were randomly assigned to receive 50, 100 or 300 mg doxycycline per day, or no medication (control group), with 15 patients in each group. In two patients in the low-dose doxycycline group, the operation had to be postponed because of full occupancy of the intensive care unit; therefore, 13 patients were evaluated in this group. Medication was started 14 days before the planned operation, and the last dose was taken in the evening before surgery. Postoperative outcome was similar in all groups.

The aneurysm sac was opened and adhering thrombus was manually removed. A tissue sample was obtained from the anterior-lateral aneurysm wall at the maximum diameter of the aneurysm. Wall samples were immediately halved. One half was snap-frozen in carbon dioxide-cooled isopentane or liquid nitrogen and stored at -80°C for later analysis. The other half was fixed in formaldehyde (24 hours), decalcified (Kristensens solution, 120 hours), and paraffin-embedded for histologic analysis. All analyses were performed in a researcher-blind fashion.

For comparison of AAA wall with nonaneurysmal wall, we used nonaneurysmal aortic wall samples from braindead kidney donors. Only patches displaying advanced atherosclerotic

lesions (equalling the characteristics of grade IV-VI lesions by the Stary classification)[22] were selected. The nonaneurysmal control group comprised 11 patients (7 men, 4 women) aged 55.6±10.2 years, with an aortic diameter of <2.0-cm. All nonaneurysmal control samples were obtained from the level of the renal artery and during a laparotomy (ie, from a comparable region and during a similar procedure as the AAA samples).

The study was approved by the Medical Ethical Committee of the Leiden University Medical Centre (LUMC), Leiden, The Netherlands.

RNA isolation and real-time quantitative polymerase chain reaction
RNA isolation and quantitative mRNA analysis by LightCycler Real-time polymerase chain reaction (PCR, Applied Biosystems, Nieuwerkerk aan den Ijssel, The Netherlands) were performed by following previously published protocols.[21] All mRNA data were standardized on basis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Tissue homogenization
Aortic wall tissues were pulverized in liquid nitrogen and homogenized in lysis buffer (10mM Tris, pH 7.0; 0.1mM calcium chloride, 0.1M sodium chloride, 0.25% (v/v) Triton X-100). This protocol releases both soluble as well as membrane-bound proteases. Samples were subsequently centrifuged at 13,000 rpm for 10 minutes at 4°C, snap-frozen, and stored at -80°C until use. Homogenates were standardized according to their protein content (Pierce, Rockford, Ill).

Specific immunocapture MMP activity assays
MMP-1, MMP-2, MMP-8, MMP-9, MMP-13, and MMP-14 activity assays (Amersham Biosciences, Buckinghamshire, United Kingdom [UK]) were performed according to the supplier's recommendations. In these assays, target proteases are captured by a specific antibody immobilized on microtiter plates, and the proteolytic activation of a modified proenzyme by the captured protease is used to quantify the activity of the captured protease. These assays allow sensitive and specific assessment of active MMPs, as well as pro-MMP upon activation of latent MMP by a mercuric salt (p-aminophenylmercuric acetate) in *in vitro* systems.

Western blot analysis
Western blot analysis was used to quantify the amount of proenzyme as well as the preceding protease activation through quantification of the amount of activated protease that was present in the tissue homogenates as protease-inhibitor complex.[18] Preliminary analyses showed that the antibodies used allow evaluation of both pro and active forms of the respective proteases and that the standard denaturing conditions required for Western blot analysis result in full dissociation of MMP–TIMP and cathepsin–cystatin C complexes, thus showing that these analyses allow assessment of inhibitor-bound MMPs and cathepsins.[18]

Western blot analyses for the proteases, as well as for cystatin C and TIMP1, were performed as previously described[23] using the antibodies antihuman MMP-2 (PC- 158, the Bindingsite,

Birmingham, UK), anti-MMP-3 (PC-112, the Bindingsite), anti-MMP-7 (RP1MMP7, Triple Point Biologics, Cambridge, UK), anti-MMP8 (MAB3316, Chemicon, Chemicon Europe Ltd, Chandlers Ford, UK), anti-MMP-9 (TNO-BEA-21, TNO, Leiden, The Netherlands), anti-cathepsin K (IM55L, Calbiochem, Breda, The Netherlands), anti-cathepsin L (AF952, R&D Systems, Abingdon, UK), anti-cathepsin S (sc-6505, Santa Cruz, Heerhugowaard, The Netherlands), anti-cystatin C (sc- 16989, Santa Cruz), and anti-TIMP1 (AB8229, Chemicon). The amount of protein in all samples was standardized using actin levels (anti-actin sc-1615, Santa Cruz).

All secondary antibodies were obtained from Santa Cruz Biotechnology (Heerhugowaard, The Netherlands). Immunoblots were visualized using Super Signal West Dura Extended Duration Substrate (Perbio Science, Etten-Leur, The Netherlands), and a luminescent image workstation (UVP, Cambridge, UK). LabWorks 4.6 software (UVP, Upland, Calif) was used to quantify the immunoblots.

Immunohistochemistry

Immunohistochemistry was performed using 4-µm deparaffinized, ethanol-dehydrated tissue sections. Sections were incubated overnight with a myeloperoxidase (A 0398 DAKO, Heverlee, Belgium), a MMP-8 (Medix Biochemica, Milsbeek, The Netherlands), or a CD68 (M 0718 DAKO) antibody. Sections were stained with Nova Red (Vector Laboratories, Burlingame, Calif) and counterstained with Mayer hematoxylin. Controls were performed by omitting the primary antibody. Specificity of myeloperoxidase staining for neutrophils was validated by morphometric analysis of myeloperoxidasepositive cells by an experienced vascular pathologist (Dr J. H. von der Thüsen). Slides were examined by two independent observers who were unaware of the patient's status.

Statistical analysis

Expression of mRNA was compared by t test or Wilcoxon Mann-Whitney U test, where appropriate. Results of the Western blots and immunohistochemistry were analyzed by Wilcoxon- Mann-Whitney U test to compare different groups. Statistical significance was accepted at a value of $P < 0.05$. Many findings in this study reflect correlated data, and as such a Bonferroni correction was *not* applied for the data in this study. Yet, a Bonferroni correction should be considered when interpreting the results of uncorrelated data. Possible dose–response relationships were evaluated by quadratic regression analysis. All analyses were performed using SPSS 12.0.1 software (SPSS Inc, Chicago, III).

Results

Patients

Clinical characteristics of the patients are compiled in the Table. All four groups were comparable with regard to age, sex, pharmaceutical treatment, and AAA diameter. Owing to the reticence toward statin use in The Netherlands, <10% of the patients in the study were receiving statin therapy. Doxycycline treatment was well tolerated, and no patients dropped out.

TABLE

Patient characteristics

Variables[a]	Control AAA AAA	Doxy 50mg	Doxy 100mg	Doxy 300mg
Number of patients	15	13	15	15
Age (y), mean(range)	74.8 (69-84)	72.7 (62-85)	74.1 (50-88)	72.1 (58-87)
AAA diameter, mean cm	6.7	6.5	6.3	6.7
Time between diagnosis and surgery, mean months	6	7	5	4
Female sex	1	2	2	3
Current smoker, No.	6	6	7	5
Medication use				
Statins	1	1	1	2
Antihypertensives	8	7	8	7
Antiplatelet therapy	10	8	8	8

[a] Values of P for all variables were not significant

Effects of doxycycline treatment on MMP expression and activation

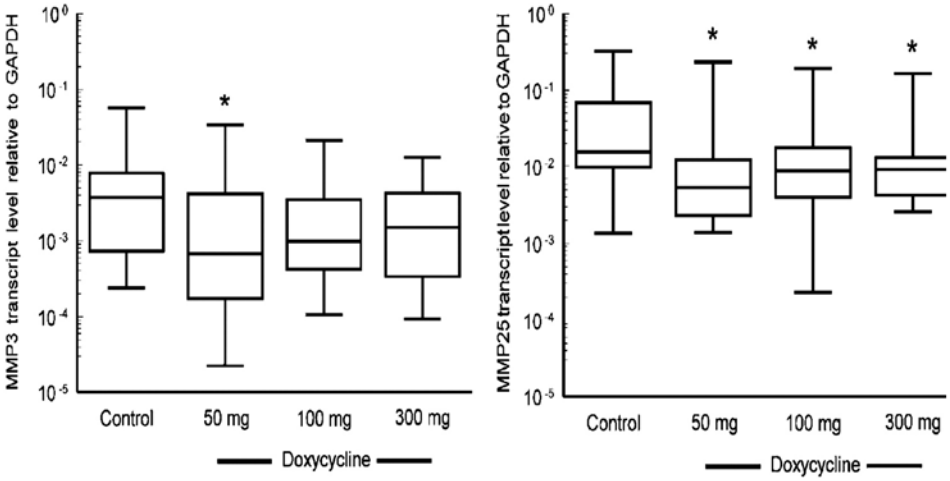
Compared with nonaneurysmal control aorta wall, AAA tissue was characterized by increased mRNA expression of soluble MMP-3 ($P < 0.029$) and MMP-9 ($P < 0.001$), as well as by abundant expression of the membrane type MMPs, MMP-16 ($P < 0.016$) and MMP-25 ($P < 0.001$). Expression of all other proteases and their inhibitors was similar in control tissue and AAA (Supplementary Table I, online only).

Figure 1 shows that independent of its dose, 14 days of preoperative doxycycline therapy attenuated MMP-3 ($P <0.045$) and MMP-25 ($P < 0.014$) mRNA expression. All three doses of doxycycline increased MMP-8 mRNA expression to values comparable to those found in control aorta (Supplementary Table II, online only). Expression of all other MMPs, including MMP-9, was not influenced by doxycycline treatment (Supplementary Table II, online only).

Possible effects of doxycycline treatment on the posttranscriptional regulation of MMP protein levels and activity were evaluated by specific protease activity assays and Western blot analyses. Direct measurement of active MMPs in aortic tissue is not feasible because of the rapid inactivation of the active enzymes by the endogenous inhibitors[18]; therefore, MMP activities in the protease activity assays were only measured after in vitro activation of latent proenzymes. Results from these assays showed a maximum level of suppression of MMP-2, MMP-8, and MMP-9 proenzyme levels in the low-dose doxycycline group (MMP-2, $P < 0.015$;

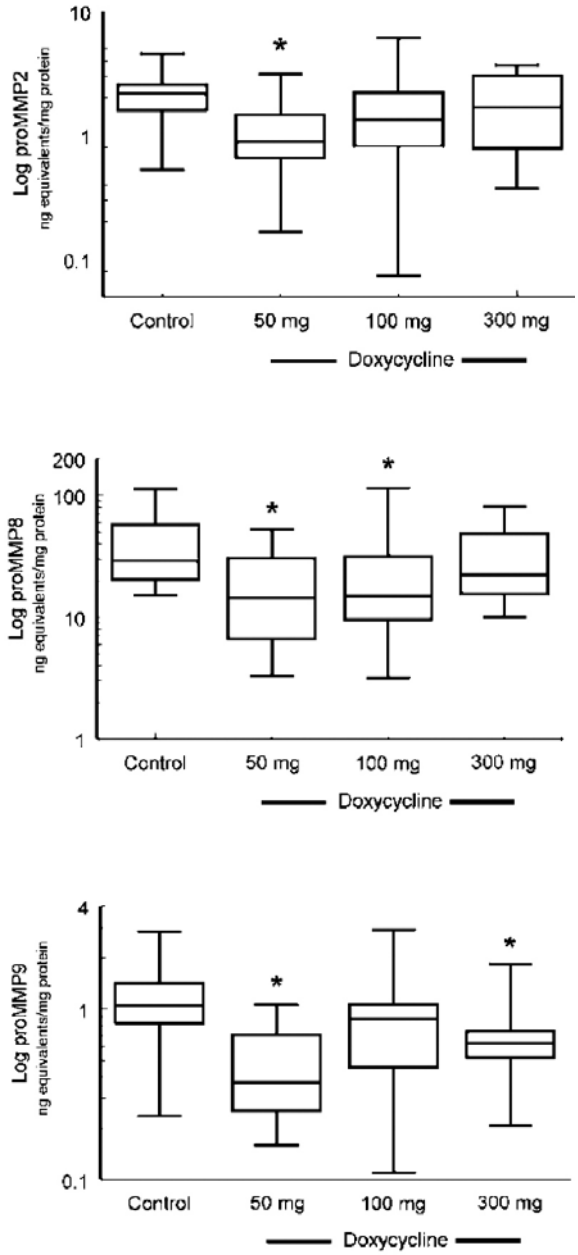
MMP-8, $P < 0.013$; and MMP-9, $P < 0.004$; Figure 2). Results for the higher doses were more variable and showed reduced aortic wall MMP-8 proenzyme levels in the standard-dose (100 mg), and reduced MMP-9 proenzyme levels in the high-dose (300 mg) doxycycline group ($P < 0.020$; Figure 2). MMP-1 and MMP-13 proenzyme levels both remained below the detection limit of the protease activity assays.

FIGURE 1



***Left,* Matrix metalloproteinase (MMP)-3 and *(right)* MMP-25 transcript levels relative to glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*. Independent of its dose, doxycycline reduces aortic wall MMP-3 and MMP-25 messenger RNA expression. Maximum level of suppression was already found in the low-dose doxycycline group. Results for the higher doses are more variable. Results are presented in box plots depicting median value *(horizontal line)*, interquartile range *(box borders)*, and range *(bars)*.**
***P* < 0.045 and *P* < 0.014 respectively (t test).**

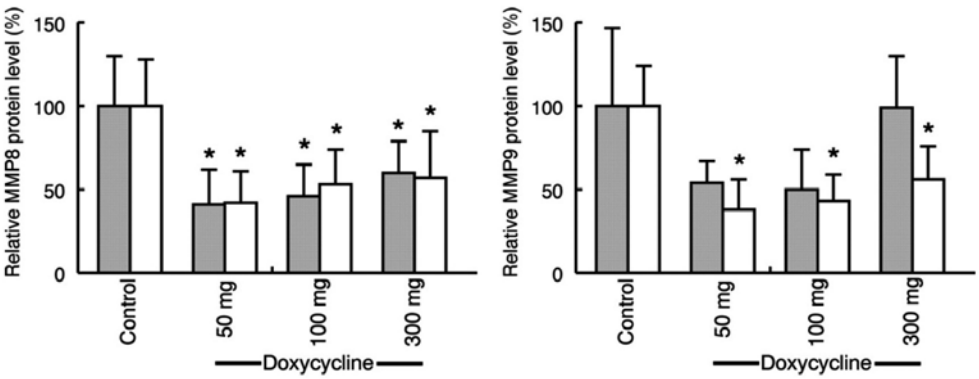
FIGURE 2



The effect of doxycycline on matrix metalloproteinase *(MMP)*-2, MMP-8, and MMP-9 proenzyme levels in untreated abdominal aortic aneurysm controls and doxycycline-treated patients as measured in the protease activity assays. Maximum level of suppression was already found in the low-dose doxycycline group. Results for the higher doses are more variable. *$P < .015$ for MMP-2, $P < 0.013$ for MMP-8, and $P < 0.004$ for MMP-9 by Wilcoxon-Mann-Whitney U test. Results are presented in box plots depicting median value *(horizontal line)*, interquartile range *(box borders)*, and range *(bars)*.

We performed Western blot analysis to evaluate a possible effect of doxycycline treatment on protease activation. Figure 3 shows that doxycycline treatment was associated with a dose-independent reduction of MMP-8 and MMP-9 *activation* ($P < 0.004$). Findings for MMP-8 proenzyme paralleled results of the protease activity assays and showed reduced MMP-8 proenzyme levels for all doses of doxycycline ($P < 0.010$). However, results for MMP-9 proenzyme were not statistically significant in the low-dose and normal-dose doxycycline groups ($P < 0.073$; Figure 3). MMP-2, MMP-3, and MMP-7 proenzyme and active enzyme levels were not influenced by doxycycline treatment (Supplementary Table III, online only). This indicates that doxycycline treatment neither influenced protein levels nor activation of these proteases.

FIGURE 3



The effect of doxycycline on pro *(gray bars)* and activated *(white bars)* (left) matrix metalloprotein *(MMP)*-8 and (right) MMP-9 levels (relative protein levels, mean control, 100%) in untreated abdominal aortic aneurysm controls (n=5) and doxycycline-treated patients (n=4 per dose) measured by Western blot analysis. Results are presented with the standard error *(range bars)*. *$P < 0.020$ by Wilcoxon-Mann-Whitney *U* test.

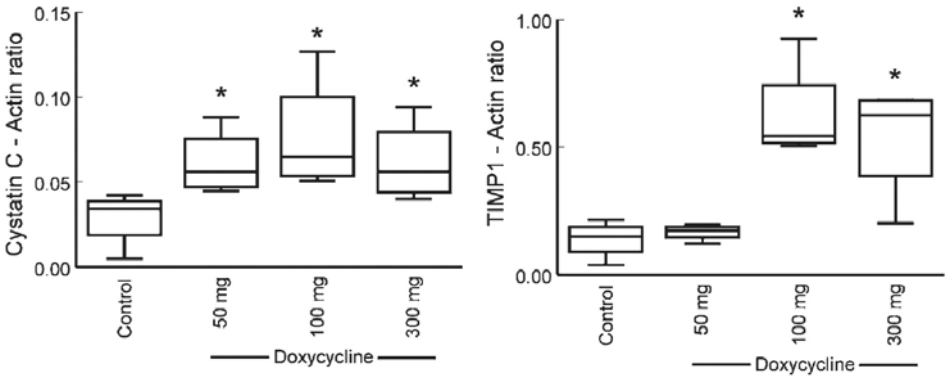
Doxycycline treatment: cysteine protease expression and activation

Doxycycline treatment for 14 days did not influence mRNA expression of the cysteine proteases cathepsin K, L, and S (Supplementary Table II, online only). Western blot analysis for pro and activated forms of cathepsin K, L, and S showed that doxycycline therapy did not influence cysteine proenzyme levels or their activation (Supplementary Table III, online only).

Doxycycline treatment: protease inhibitor expression

With the exception of a moderate reduction of TIMP2 mRNA expression ($P < 0.004$), none of the doxycycline doses influenced expression of the protease inhibitors (Supplementary Table II, online only). Yet, as shown in Figure 4, doxycycline therapy increased cystatin C and TIMP1 protein levels (TIMP1 normal-dose and high-dose doxycycline only, $P < 0.029$).

FIGURE 4

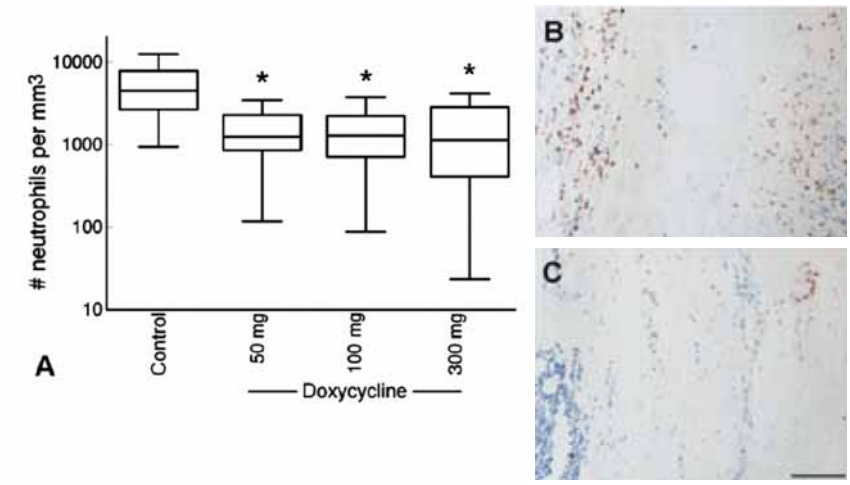


Doxycycline treatment increases protein levels of *(left)* cystatin C, the principle extracellular inhibitor of cysteine proteases, as well as (right) tissue inhibitor of metalloproteinase 1 *(TIMP1)* protein levels (100 and 300 mg only). Results are presented in box plots depicting median value *(horizontal line)*, interquartile range *(box borders)*, and range *(bars)*. *$P < 0.029$ by Wilcoxon-Mann-Whitney *U* test.

Doxycycline treatment reduces neutrophil influx in the aneurysmal wall.

The rather selective reduction of MMP-8 and MMP-9 (neutrophil collagenase and gelatinase, respectively) protein levels—but not of their mRNA expression—along with reduced mRNA expression of MT6-MMP, the neutrophil-specific MMP-25,[24] suggested that the observed effects of preoperative doxycycline treatment on MMP expression are indirect and may relate to a reduced aortic wall neutrophil content. We quantified the aortic wall neutrophil content by immunohistochemistry (myeloperoxidase staining, Figure 5) that indeed showed that 2 weeks of preoperative doxycycline treatment resulted in a profound reduction of the neutrophil content (Figure 5, $P < .001$). Specificity of myeloperoxidase (Figure 5, B and C) staining for neutrophils was validated by morphometric analysis of myeloperoxidase-positive cells and absence of an effect of doxycycline on myeloperoxidase mRNA expression and on aortic wall macrophage content (Supplementary Fig, online only). The findings for myeloperoxidase staining were validated by MMP-8 staining, which showed similar results as the myeloperoxidase staining (data not shown).

FIGURE 5



A, Doxycycline reduces neutrophil content in the aneurysmal wall. Results are presented in box plots depicting median value *(horizontal line)*, interquartile range *(box borders)*, and range (bars). **P* < 0.001 by Wilcoxon-Mann- Whitney *U* test. B, Aneurysm wall neutrophil infiltration (myeloperoxidase staining) in the medial-adventitial border zone in untreated and (C) doxycycline-treated individuals. *Bar* represents 100 µm

Discussion

Doxycycline has been shown convincingly to prevent AAA formation in a variety of animal models,[10-13] and the results from two small clinical studies suggest that doxycycline may also reduce AAA expansion in people.[14,15] Remarkably, although the rationale behind doxycycline therapy is based on its putative effects on MMP-9 expression and activity,[9] the effects of doxycycline on MMPs in the *human* aneurysm are unclear, with published studies[17,19] suggesting that doxycycline acts through a different mechanism.[16]

In this prospective clinical trial, we examined the effect of three pharmacologically relevant doses of doxycycline—low (50 mg/d), regular (100 mg/d) or high (300 mg/d)—on inflammatory processes in the aneurysmal wall of patients scheduled for elective, open AAA repair in an integrative approach. We first established mRNA expression profiles of the MMP and cysteine collagenases by quantitative real-time PCR. Because this approach does not provide information on the post-transcriptional regulation of protease activity, we applied specific protease activity assays and Western blot analyses to address the post-translational regulation of protease activity, and quantified tissue expression of specific inhibitors of proteases.

Preoperative doxycycline treatment resulted in a selective reduction of MMP-8 and MMP-9 protein levels, as well as suppression of MMP-25 mRNA expression. The effect on MMP-8 and MMP-9 protein levels, but not on mRNA expression, along with the rather selective effect on MMP-25 mRNA expression,[24] suggests that the effect of doxycycline on protease

expression was indirect and may relate to suppression of neutrophil influx. Neutrophil MMP-8 and MMP-9 are both stored (secondary granule) proteins that are temporarily expressed during the late myeloid maturation pathway of neutrophils and are minimally expressed in mature neutrophils.[25,26] This could well account for the apparent discrepancy between the effects of doxycycline therapy on MMP-8 and MMP-9 protein and mRNA expression.

We evaluated a possible effect on neutrophil influx in the aneurysm wall by immunohistochemical staining for myeloperoxidase, the preferred neutrophil marker (Prof D. Roos, Sanquin Research, Amsterdam, The Netherlands, personal, communication) and indeed found that doxycycline treatment profoundly reduced the number of myeloperoxidase-positive cells. The specificity of the myeloperoxidase staining for neutrophils was confirmed by morphometric analysis that showed that all myeloperoxidasepositive cells exhibit a lobed nucleus, typical for neutrophils, and by MMP-8 staining that showed analogous results to the myeloperoxidase staining.

Our immunohistochemical analysis did not indicate myeloperoxidase expression in monocytes and or macrophages.[27] To exclude possible interference by an effect of doxycycline on monocyte/macrophage content or their myeloperoxidase expression, we quantified the number of CD68-positive cells and assessed myeloperoxidase mRNA expression, both of which remained unaffected by doxycycline therapy (Supplementary data, online only).

Our observations are remarkable and suggest that doxycycline may act through an effect on neutrophil infiltration. Neutrophil abundance (presumably resulting from interleukin-8 hyperexpression) is a striking characteristic of AAA disease,[28] and neutrophils may be critically involved in aneurysm formation and growth. Eliason et al[29] and Pagano et al[30] recently showed that abrogated neutrophil influx forestalls aneurysm formation in the elastase model of aneurysm formation, indicating that neutrophil influx is critically involved in aneurysm formation in this established model of aneurysm formation. The pathophysiologic role of the neutrophils in AAA formation and aneurismal growth is still unclear. Eliason et al[29] showed that the effect occurred independently from MMP-8. Our findings of increased TIMP1 and cystatin C protein levels upon doxycycline therapy provide an alternative explanation: we previously showed that cystatin C deficiency in AAA is secondary and relates to proteolytic degradation by neutrophilderived proteases.[18] An analogous mechanism has been described for TIMP1,[31] suggesting that neutrophils may unfavorably influence the proteolytic balance through an effect on the inhibitors of protease activity.

Doxycycline has been shown to inhibit neutrophil migration in in vitro studies and studies in healthy volunteers[32]; but to our knowledge, this is the first clinical study showing that doxycycline treatment results in a significantly reduced neutrophil influx in a chronic inflammatory condition such as AAA. The mechanism underlying the reduced neutrophil influx is yet unclear. Tetracyclines, including doxycycline, are known for their pleiotropic immunomodulatory activities that include suppression of inducible nitric oxide synthase and cyclooxygenase-2 expression.[33] Both pathways are implicated in the perpetuation of

the inflammatory processes of AAA,[34,35] suggesting that doxycycline treatment may act by attenuation of vascular inflammation. Sugita et al,[36] on the other hand, showed that doxycycline treatment by chelation of intracellular Ca++-ions resulted in a dose-dependent inhibition of neutrophil chemotaxis. Reductions in aortic wall neutrophil content in patients in this study occurred independently of the dose and were already maximal at the sub-antimicrobial dose of doxycycline, suggesting that the effect relates to an anti-inflammatory mechanism.

Independent of its effects on neutrophil influx, doxycycline may further act through direct inhibition of MMP activity (zinc scavenging).[37] Presumably, this effect is dosedependent and thus more prominent at higher doses. Unfortunately, such binding is lost during the washing steps required in MMP activity assays and not recognizable in Western blot analysis, and we are therefore unable to address the relevance of such an effect.

Conclusion

Preoperative doxycycline therapy for 14 days resulted in reduced aortic wall neutrophil content that is accompanied by a selective suppression of neutrophil-derived proteases and increased protein levels of the protease inhibitors TIMP1 and cystatin C. Results from this study provide a rationale for the evaluation of doxycycline as a therapeutic means of inhibition of aneurysmal growth. Moreover, our observations argue for a broader application for doxycycline therapy in other pathologies involving neutrophil infiltration and aneurysm formation, such as Kawasaki disease and Behçet disease,[38,39] as well as nonvascular conditions with prominent neutrophil involvement such as Sweet syndrome[40] and chronic obstructive pulmonary disease.[41]

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Enhanced expression and activation of pro-inflammatory transcription factors distinguish aneurysmal from atherosclerotic aorta: IL-6- and IL-8- dominated inflammatory responses prevail in the human aneurysm

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Abstract

Inflammation plays a key role in the pathogenesis of an AAA (abdominal aortic aneurysm); however, the nature of the inflammatory factors and cellular response(s) involved in AAA growth is controversial. In the present study, we set out to determine the aortic levels of inflammatory cytokines in relation to downstream inflammatory transcription factors and cellular responses. A comparison of AAA wall samples with atherosclerotic wall samples taken from the same aortic region allowed AAA-specific inflammatory parameters to be identified that distinguish AAAs from ASD (aortic atherosclerotic disease). RT-PCR (real-time PCR), ELISA, Western blotting and immunohistochemistry were combined to assess cytokines and transcription factors at the mRNA and protein level, and their activation status. Compared with ASD, inflammatory parameters associated with Th1-type [T-bet, IL (interleukin)-2, IFN-γ (interferon-γ), TNF-α (tumor necrosis factor-α), IL-1α and cytotoxic T-cells] and Th2-type [GATA3, IL-4, IL-10, IL-13 and B-cells] responses were all increased in AAA samples. Evaluation of major downstream inflammatory transcription factors revealed higher baseline levels of C/EBP (CCAAT/enhancer-binding protein) α, β and δ in the AAA samples. Baseline p65 NF-κB (nuclear factor κB) and c-Jun [AP-1 (activator protein-1)] levels were comparable, but their activated forms were strongly increased in the AAA samples. Downstream target genes of p65 NF-κB, c-Jun, IL-6 and IL-8 were hyperexpressed.

Molecular and cellular processes associated with IL-6 and IL-8 hyperactivation were enhanced in the AAA samples, i.e. the expression of phospho-STAT-3 (signal transducer and activator of transcription-3) and perforin were elevated, and the content of plasma cells, neutrophils and vasa vasorum was increased. In conclusion, our findings demonstrate that an AAA is a general inflammatory condition which is characterized by enhanced expression and activation of pro-inflammatory transcription factors, accompanied by IL-6 and IL-8 hyperexpression and exaggerated downstream cellular responses, which together clearly distinguish an AAA from ASD.

Introduction

An AAA (abdominal aortic aneurysm) is a focal balloon-like dilation of the terminal aortic segment. The atherosclerotic aneurysm, the common fusiform form of an AAA, is a frequent pathology and a major cause of death due to rupture[1]. The hallmark pathology of an atherosclerotic AAA is a chronic inflammatory condition of the arterial wall that is accompanied by a proteolytic imbalance[2]. Increased proteolytic activity results in excessive matrix degradation and progressive weakening of the vessel wall[3]. Despite considerable knowledge of the pathomorphology of AAAs, understanding the cellular and molecular inflammatory processes which underlie the increased protease expression and that drive aneurysmal growth is limited[3]. Available human studies analyzing the cellular composition of AAAs indicate an extensive heterogeneous inflammatory response that involves macrophages, neutrophils and cytotoxic T-cells as well as Th1 and Th2 (T-helper 1 and 2) cell subsets[4–6].

It is well established that the Th1-/Th2-type balance is controlled tightly, and that exaggerated Th1 or Th2 responses are causatively associated with the development of

inflammatory pathologies[7,8]. Th1- and Th2- type cellular responses involve distinct (and partly even opposite) immune and inflammatory processes, which are mediated by specific sets of cytokines and transcription factors. Although Th1-type responses are typically associated with enhanced expression of the cytokines IL (interleukin)-2 and IFN-γ (interferon-γ) and the transcription factor T-bet, Th2-type responses are characterized by a dominance of IL-4, IL-5 and/or IL-10 and elevated levels of the transcriptional regulator GATA3[9].

In the context of a developing AAA, the Th1/Th2 balance is thought to play an important regulatory role in the control of matrix remodeling and inflammatory processes relevant for AAA growth and rupture[3]. However, the nature of the predominant cellular response (Th1 or Th2) and the inflammatory factors predominantly involved in human AAA growth is controversial. For example, Galle and co-workers[10] reported a predominance of Th1-type cellular and cytokine responses, whereas other studies have demonstrated the predominance of a Th2-type immune response[11], which is reflected by the enhanced expression of Th2 associated cytokines and minimal expression of the Th1-associated cytokine IFN-γ[3,11]. A recent protein array study assessing the cytokine and chemokine profile of aortic wall tissue from advanced (>5.5 cm) AAAs has reported the up-regulation of both Th1-associated [IL-1 and TNF-α (tumor necrosis factor-α)] and Th2- associated (IL-10) inflammatory mediators[12]. The downstream consequences of these and similar[13, 14] observations remain uncertain as, at the transcription factor level, cytokines can exert opposite effects. For example, IL-1 can activate NF-κB (nuclear factor κB), whereas IL-10 can suppress its activation.

With a comprehensive analysis of transcription factors lacking in the human studies performed so far, it remains unclear whether, and if so how, the observed changes in cytokine expression in the human AAA wall affect the basal expression of major inflammatory transcription factors and their degree of activation.

In the present study, we set out to address this issue and characterized AAA samples >5.5 cm with respect to their cytokine expression profile (at the mRNA and protein levels) and their cellular composition (using immunohistochemistry). We subsequently analyzed the basal expression levels of major inflammatory transcription factors [p65 NF-κB, c-Jun, C/EBP (CCAAT/ enhancer-binding protein) α/β/δ and STAT-3 (signal transducer and activator of transcription-3)] and their activation status (active p65 NF-κB, phospho-c-Jun and phospho-STAT-3) in relation to relevant inflammatory processes controlled by these transcription factors, i.e. the expression of putative target genes (IL-6 and IL-8) and their associated cellular effects (B cell, plasma cell and neutrophil content, and perforin expression)

Because a putatively exaggerated Th2 response in an AAA would clearly distinguish this pathology from ASD (aortic atherosclerotic disease), which is governed by Th1-type cellular and cytokine responses[3], a comparison of AAAs with ASD was considered relevant. The more so as AAAs and ASD share common pathological features and risk factors, but differences in their pathogenesis are only poorly defined to date[3].

The results of the present study demonstrate elevated levels of the three C/EBP isoforms (α, β and δ), and comparable basal but enhanced activated levels of the inflammatory transcription factors NF-κB and c-Jun in the AAA wall. These findings at the transcription factor level are in accordance with hyperexpression of downstream genes, in particular the cytokines IL-6 and IL-8, and subsequent functional effects at the molecular (increased phospho-STAT-3) and cellular (increased plasma cell and cytotoxic T-cell content) level. Taken together, these differences characterize AAAs as an inflammatory condition and clearly distinguish them from ASD.

Materials and Methods

Patients

All human arterial wall samples were provided by the Vascular Tissue Bank (Department of Vascular Surgery, Leiden, The Netherlands). Sample collection and handling was performed in accordance with the guidelines of Medical Ethical Committee of the Leiden University Medical Center, Leiden, The Netherlands. All samples were obtained following consent of the patients. None of the patients in the present study had a history of diabetic or chronic inflammatory disease. The primary cause of the fatal brain injury in the ASD group was a major head trauma or subarachnoidal bleeding.

Anterior lateral aneurysm wall samples were obtained from patients with an AAA >5.5 cm undergoing elective open repair (AAA group: *n*=17; age, 72.4+− 6.2 years (value is the mean+− S.D.); 14 males/three females; and AAA diameter, 6.7+− 1.1 cm). Due to reticent statin use only one patient with an AAA used a statin. For comparison of the AAA wall with the atherosclerotic aortic wall, we used non-aneurysmal aortic wall samples from brain-dead kidney donors. Only patches displaying advanced atherosclerosis with advanced atherosclerotic lesions (equaling the characteristics of grade IV– VI lesions according to the Stary classification [15]) were selected (ASD group; *n*=11; age, 55.6+− 10.2 years; seven males/four females; aortic diameter, <2.0 cm). Of note, all ASD samples were obtained at the level of the renal artery and during a laparotomy, i.e. from a comparable region and during a similar procedure as the AAA samples.

Any adhering thrombus was carefully removed and the aortic walls (either AAA or ASD) were divided in two. One half was immediately snap-frozen in liquid nitrogen and stored at −80 ◦C for mRNA [RT-PCR (real-time PCR)] and protein (Western blot, Multiplex and ELISA) analysis. The other half was fixed in formaldehyde for 24 h, decalcified using Kristensen's solution for 120 h and embedded in paraffin for histological analysis.

RNA extraction and mRNA analysis

Total RNA extraction was performed using RNAzol (Campro Scientific) and glass beads according to the manufacturer's instructions. Subsequently, cDNA was prepared using a Promega kit for RT-PCR. The mastermix (Eurogentec), an ABI-7700 system (Applied Biosystems) and established primer/probe sets (Applied Biosystems; Table 1) were used according to the manufacturer's instructions and as reported previously[16]. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a reference and for normalization.

TABLE 1

T-bet	Hs00203436_m1
GATA-3	Hs00231122_m1
IL-1α	Hs00174092_m1
IL-1β	Hs00174097_m1
IL-2	Hs00174114_m1
IL-4	Hs00174122_m1
IL-8	Hs00174103_m1
IL-10	Hs00174086_m1
IL-13	Hs00174379_m1
IFN-γ	Hs00174143_m1
TNF-α	Hs00174128_m1
MCP-1	Hs00234140_m1
MIP-1β	Hs99999148_m1
TGF-β	Hs99999918_m1
IL-6	Forward, 5_-TGTAGCCGCCCCACACA-3'
	Reverse, 5_-AGATGCCGTCGAGGATGTACC-3'
	Probe, 5_-FAM-AGCCACTCACCTCTTCAGAACGAATTGACA-3'
MIF	Forward, 5_-TGGCCGAGCGCCTG-3'
	Reverse, 5_-GCCGCGTTCATGTCGTAATA-3'
	Probe, 5_-FAM-CATCAGCCCGGACAGGGTCTACATCA-3'

TaqMan® gene expression assays (Applied Biosystems) or probe/primer combinations (IL-6 and MIF) used in RT-PCR
FAM, 6-carboxyfluorescein; TGF-β, transforming growth factor-β

Tissue homogenization for protein analysis

Aortic wall tissues were pulverized in liquid nitrogen and homogenized in 2 voles of lysis buffer [10 mmol/l Tris/HCl (pH 7.0), 0.1 mmol/l CaCl2, 0.1 mol/l NaCl and 0.25%TritonX-100]. This protocol releases both soluble as well as membrane-bound proteins. Samples were subsequently centrifuged at 10 000 g for 15 min at 4◦C, snapfrozen in liquid nitrogen and stored at −80◦C until use. Protein content in the homogenates was determined with a BCA (bicinchoninic acid) protein assay kit (Pierce).

Multiplex assay and ELISAs

IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte/macrophage colony-stimulating factor), IFN-γ ,MCP-1 (monocyte chemoattractant protein-1), MIP-1β (macrophage inflammatory protein-1β) and TNF-α protein levels in tissue homogenates were determined using a Bio-Plex 17 panel for multiple cytokines (Bio-Rad Laboratories). Detection thresholds for the cytokine panel was <0.5 pg/ml for IL-1, IL-2, IL-5, IL-6, IL-7, IL-8, IL-10 and TNF-α, <1 pg/ml for IL-12 and IL-17A, and <5 pg/ml for IL-4, IL-13, G-CSF, GM-CSF, IFN-γ and MCP-1. IL-6, IL-8 and MCP-1 levels exceeded the upper detection limit of the Bio-Plex[17] panel and were therefore determined

accurately using separate specific ELISAs [PeliKane compact kit (Sanquin Reagents) for IL-6 and IL-8 and a Quantikine kit (R&D Systems) for MCP-1]. sIL-6R [soluble IL-6R (IL-6 receptor)] levels were determined using an ELISA specific for human sIL-6Rs (R&D Systems).

Western blot analysis

Western blot analysis was performed essentially as described previously [17] using antibodies specific for human p65 NF-κB (both non-active and active; Santa Cruz Laboratories and Chemicon respectively), c-Jun (Santa Cruz Laboratories), phospho-c-Jun (Ser73) (Santa Cruz Laboratories), perforin (SantaCruzLaboratories), STAT- 3 (Santa Cruz Laboratories), phospho-STAT-3 (Epitomics), C/EBPα (Santa Cruz Laboratories), C/EBPβ (Santa Cruz Laboratories), C/EBPδ (Santa Cruz Laboratories) and β-actin (Santa Cruz Laboratories) as described previously [18].

The corresponding secondary antibodies were obtained from Santa Cruz Biotechnology [donkey anti- (goat IgG)] and Pierce [goat anti-(rabbit IgG) and goat anti-(mouse IgG)]. Immunoblots were visualized and quantified using the Super Signal West Dura Extended Duration Substrate (Perbio Science), LabWorks 4.6 software and a luminescent image workstation (UVP). Protein expression levels in aortic wall samples were normalized for β-actin, and separate anti-(β-actin) immunoblots were performed for each sample.

Immunohistochemistry

Immunohistochemistry was performed using deparaffinized ethanol-dehydrated tissue sections (4 μm thick) essentially as described previously [16, 19]. Sections were incubated overnight with polyclonal antibodies specifically staining human MPO (myeloperoxidase; 1:4000 dilution; DAKO), CD20 (1:1000 dilution; DAKO), CD138 (1:1000 dilution; Serotec), IL-6 (1:2000 dilution; Biogenesis) or IL-8 (1:200 dilution; Biogenesis). Conjugated biotinylated anti-(goat IgG) or anti-(rabbit IgG) were used as secondary antibodies. Sections were developed with Nova Red® (Vector Laboratories) and counterstained with Mayer's haematoxylin to allow morphological analysis. The specificity of the antibody staining was confirmed by omitting the primary antibody. The presence of eosinophils was evaluated morphologically by haematoxylin/eosin staining.

Statistical analysis

All values are expressed as means+− S.D. for normally distributed data or medians (range) for non normally distributed data. Normally distributed continuous variables were analyzed using a Student *t* test, whereas non-normally distributed continuous data were analyzed with a Wilcoxon–Mann–Whitney test using (SPSS 11.5 for Windows). CIA (Confidence Interval Analysis; version 2.0.0. 41; https://www.som.soton.ac.uk/cia/) was used to calculate the non-parametric 95% CI (confidence interval) for differences in mRNA expression. Possible relationships between aneurysm diameter and inflammatory markers were evaluated by Pearson's correlation test. The level of statistical significance was set at $P<0.05$. The present study incorporates multiple statistical comparisons. For the sake of clarity, non-corrected data are provided; however, a Bonferroni correction should be considered when interpreting non-correlated data.

Results

Characterization of aneurysmal and atherosclerotic aortic walls based on cytokine expression profiles

Aortic aneuysmal (AAA) and atherosclerotic (ASD) wall samples used in the present study were characterized for the expression of markers of inflammation. RTPCR and the Bio-Plex assay were used to determine the expression level of specific Th1-associated, Th2- associated and general inflammatory factors on the mRNA and/or protein level.

Compared with ASD, the AAA group had significantly higher aortic mRNA expression levels of the Th1- associated transcription factor T-bet (24-fold increase) and the Th2-associated transcription factor GATA3 (12-fold increase; Table 2). The T-bet/GATA3 ratio was similar in both experimental groups (P=0.52).

A subsequent analysis of cytokines in aortic mRNA extracts revealed substantially increased mRNA levels of the Th1-associated cytokines IL-2, IFN-γ, TNF-α, IL- 1α and IL-1β in AAA samples. In line with this observation, the protein levels of IL-2, IFN-γ, TNF-α and IL-1α were significantly higher in AAA homogenates (Table 3).

With respect to Th2-associated cytokines, higher mRNA levels of IL-4, IL-10 and IL-13 ($P<0.01$) were found in the AAA samples (Table 2). An increase in IL-4 and IL-13 protein levels was also observed, whereas IL-5 and IL-10 levels were below the detection limit of the Bio-Plex assay (Table 3).

In addition to these typical Th1- and Th2-associated factors, cytokines/chemokines reflecting a general pro-inflammatory status, such as MCP-1, MIP-1β, MIF (migration inhibitory factor), G-CSF and GM-CSF, were also significantly expressed in the AAA samples compared with the ASD samples (at the mRNA and/or protein level; Tables 2 and 3).

Taken together, the above molecular characterization of aortic wall samples by cytokine profiling does not indicate a clear Th1/Th2 polarization in AAAs, but points to a generic enhancement of inflammation compared with ASD.

TABLE 2

	fold increase in AAA	*P value*	ΔCt AAA	ΔCt ASD
Th1-associated				
T-bet	24.3	≤0.05	8.7 [4.3-20]	13.3 [5.2-20]
IL-2	8.6	<0.001	9.7 [6.0-13.3]	16.2 [10.7-20]
Interferon-γ	630	<0.001	9.0 [5.8-13.5]	18.3 [8.6-20]
TNF-α	2.3	0.09	8.1[3.9-11.1]	9.3 [6.0-20]
IL-1α	315.2	<0.01	8.3 [4.0-20.0]	16.6 [5.7-20.0]
IL-1β	11.3	<0.001	2.7 (1.5)	6.2 (2.8)
Th2-associated				
GATA-3	12.1	<0.01	6.8 [3.1-11.4]	10.4 [5.4-20]
IL-4	1.7	0.12	1.9 [7.1-15.2]	12.7 [9.9-20]
IL-10	1.4	0.55	5.8 [3.2-9.4]	6.3 [3.3-20]
IL-13	512	<0.01	11.0 [4.8-20]	20.0 [11.8-20]
General inflammatory				
MCP-1	3.2	<0.05	-1.7 (1.8)	0.0 (1.7)
MIP-1β	3.7	<0.05	1.2 (1.3)	3.1 (3.0)
MIF	2.1	<0.01	-2.0 (1.2)	-0.9 (0.9)
TGF-β	1.3	0.19	-0.9 (0.9)	-0.5 (1.1)

Fold differences in gene expression of markers of Th1-associated, Th2-associated and general inflammatory responses

mRNA expression was analyzed in aneurysmal wall (AAA; n =17) and atherosclerotic wall (ASD; n =11) samples. Gene expression level of the measured genes in ASD was set at 1, and relative gene expression levels in AAA tissues are expressed as the median fold increase, together with the corresponding 95 % CI of the median fold increase and the P value. The level of mRNA expression for each gene of interest was calculated using Ct values. Ct values are defined as the number of PCR cycles at which the fluorescent signal generated during the PCR reaches a fixed threshold. For each sample, the Ct for the target gene and for the housekeeping gene was determined to calculate ΔCt (Ct, target gene ΔCt, housekeeping gene). The relative expression of a gene can be calculated from the formula 2–ΔCt. The corresponding ΔCt values for AAA and ASD are means ± S.D. (for normally distributed data) or medians (range) (for non-normally distributed data). High (low) ΔCt values reflect low (high) mRNA expression levels. A ΔCt value of 20 represents the detection limit of the assay. TGF-β, transforming growth factor-β.

Characterization on the basis of cellular markers of Th1/Th2 polarization

To characterize aneurysmal (AAA) and atherosclerotic (ASD) aortas further, their cellular composition was evaluated with particular emphasis on Th1- and Th2-associated cellular responses.

Absolute and relative cytotoxic T-cell infiltration (Th1-associated) was assessed by analyzing aortic CD4 and CD8 mRNA expression levels 20. AAA samples had higher CD8 (26 fold; P<0.001) and CD4 (12-fold; P=0.24) mRNA expression levels and a higher CD8/CD4 ratio

TABLE 3

Protein expression (ng/mg of total aortic protein)

	AAA	ASD	*P value*
Th1-associated			
IL-2	0.3 [0.04-0.6]	ND	<0.001
Interferon-γ	8.2 [2.3-14.9]	0.0 [0-4.4]	<0.001
TNF-α	0.2 [0.0-0.8]	ND	<0.001
IL-1β	4.3 [1.1-19.1]	0.42 [0.00-	<0.001
Th2-associated			
IL-4	0.3 [0-1.4]	ND	0.12
IL-5	ND	ND	NA
IL-10	ND	ND	NA
IL-13	1.4 [0.3-5.4]	ND	<0.01
General inflammatory			
MCP-1	2 13 [85-1781]	116 [48-923]	0.17
MIP-1β	7.5 [5.1-27.4]	7.0 [0.5-14.2]	0.23
G-CSF	6.0 [1.3-31.4]	0.0 [0-0.75]	<0.001
GM-CSF	0.3 [0-1.5]	ND	<0.01
IL-7	0.2 [0-0.6]	ND	<0.01
IL-12	<0.05 [0-0.3]	ND	0.12
IL-17	<0.05 [0-11.7]	ND	0.53

Differences in protein expression of markers of Th1-associated, Th2-associated and general inflammatory responses

Aortic wall protein expression levels of markers of Th1-associated, Th2-associated and general inflammatory responses were determined in aneurysmal wall (AAA; n =14) and atherosclerotic wall (ASD; n =11) samples using a Bio-Plex assay or ELISA for MCP-1. Values are medians (range). ND, not detectable (i.e. below the detection limit of the Bio-Plex assay). NA, not applicable.

(P<0.001), suggesting an enhanced influx of cytotoxic T-cells in AAA tissue. Specific IHC (immunohistochemical) staining of cytotoxic T-cells in sections prepared from AAA samples confirmed an increased abundance of cytotoxic T-cells compared with ASD samples (results not shown).

Potential Th2-related cellular effects were evaluated by IHC staining of B-cells (CD20) and an assessment of eosinophil infiltration. AAA samples had a dispersed CD20 immunoreactivity, which was infrequently observed in ASD samples (limited to infiltrates in the medial-adventitial transition zone in some ASD controls), clearly demonstrating enhanced B-cell infiltration in AAAs (Figures 1A and 1B). Eosinophils were not found in AAA or ASD samples (results not shown).

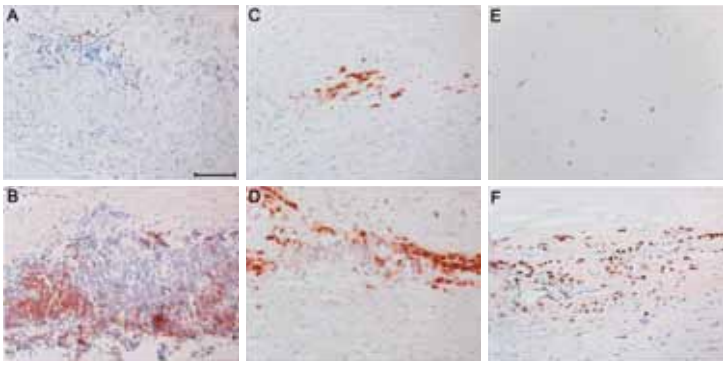
The results of our cellular characterization are consistent with the cytokine expression findings and demonstrate the over-representation of cells important for Th1- and Th2-type responses. Subsequently, the downstream consequences were examined by assessing the basal expression level and/or the activation status of relevant inflammatory transcription factors, i.e. transcription factors mainly involved in the signal transduction of the cytokines expressed in AAAs{C/EBPα,C/EBPβ and C/EBPδ,NF-κB and c-Jun [AP-1 (activator protein-1)]}.

Enhanced expression of (activated) pro-inflammatory transcription factors in AAAs
Protein homogenates of AAA and ASD samples were prepared and subjected to Western blot analysis. Aortic transcription factor concentrations were quantified relative to β-actin. When compared with the ASD samples, the aortic concentrations of the C/EBPα, C/EBPβ, C/EBPδ isoforms were 6.3-, 3.1- and 4.2-fold higher ($P<0.05$) respectively, in the AAA samples (Figure 2A). Baseline p65 NF-κB and c-Jun expression levels were comparable in the AAA and ASD samples (Figure 2B), but the aortic wall concentrations of their activated forms, p65 NF-κBactive and phospho-c-Jun, were 3.7- and 3.3-fold (both $P<0.001$) higher respectively, in the AAA samples as assessed by antibodies specifically detecting activated/ phosphorylated epitopes (Figure 2B). Taken together, these results demonstrate an enhanced pro-inflammatory status at the transcription factor level in AAAs, despite enhancement of expression of cytokines with anti-inflammatory properties (e.g. IL-10).

Enhanced IL-6 and IL-8 expression in AAAs
Next, we examined whether inflammatory factors that are positively regulated by C/EBPs, p65 NF-κB and c-Jun are also elevated in AAAs when compared with ASD. IL-8 transcription and IL-6 expression rely on the concomitant activation of signaling pathways activating NF- κB and AP-1 (i.e. c-Jun) or NF-κB and C/EBPs[21,22]. Indeed, IL-6 and IL-8 mRNA expression was strongly and significantly elevated in AAA samples compared with ASD samples (Table 4). Analysis of IL-6 and IL-8 expression levels in AAA and ASD tissue homogenates by ELISA confirmed that the AAA samples contained very large amounts of IL-6 and IL-8 (Figure 3A). IL-6 and IL-8 levels exceeded the levels of all other cytokines determined in the AAA samples and were increased more than 100-fold ($P<0.001$) compared with the ASD samples. No relationship was found between the IL-6 or IL-8 level and the aneurysm diameter.

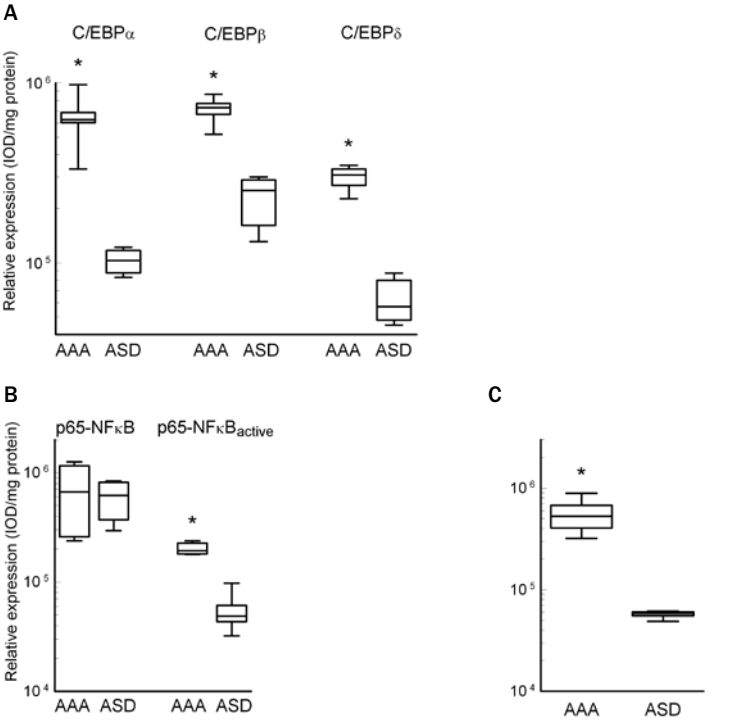
Hyperexpression of IL-6 and IL-8 clearly demonstrates an inflammatory divergence between AAAs and ASD. IHC analysis of IL-6 and IL-8 expression (Figure 3B) confirmed the abundant expression of both cytokines in AAA samples. Of note, IL-6 and IL-8 immunoreactivity in AAA samples were dispersed throughout all layers of the aortic wall and not essentially confined to the intimal layer and intimal border zone of the media as in ASD samples. Refined analysis of the cell types expressing IL 6 and IL-8 in AAA samples revealed that IL-6 immunoreactivity was predominantly associated with plasma cells and macrophages, whereas IL-8 immunoreactivity was mainly associated with lymphocytes and neutrophils. IL-6 and IL-8 immunoreactivity in ASD samples on the other hand was predominantly limited to foam cells/ macrophages, vascular smooth muscle cells and lymphocytes. Taken together, these results demonstrate a transmural hyperexpression of IL-6 and IL-8 in AAAs.

FIGURE 1



IHC analysis of the cellular composition of AAA and ASD tissue. Representative photomicrographs (similar magnification) of ASD (above) and AAA (under) tissue stained with antibodies specifically detecting (A and B) B-lymphocytes (B-cells; anti-CD20), (C and D) plasma cells (anti-CD138), and (E and F) neutrophils (MPO). Scale bar, 100 μm.

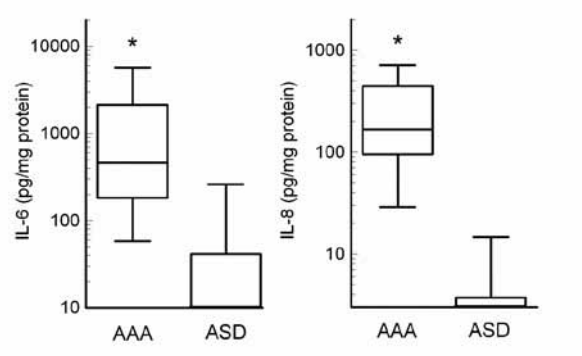
FIGURE 2



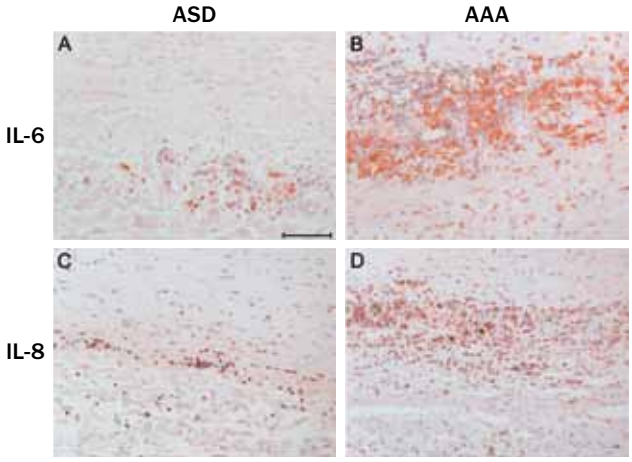
Elevated aortic concentrations of inflammatory transcription factors are characteristic of the AAA wall. Basal levels of (A) C/EBPα, C/EBPβ and C/EBPδ, and (B) p65 NF-κB, c-Jun protein and their activated forms [NF-κBactive and phospho-c-Jun (p-c-Jun)] were determined by Western blotting in homogenates of AAA and ASD samples. Transcription factor expression was determined relative to β-actin for each sample. Results are presented in boxplots (median value is indicated by the solid horizontal line, the lower and upper quartiles are indicated by the box, and the range is indicated by the error bars). *P <0.001 compared with ASD. IOD, integrated optical density.

FIGURE 3

Elevated aortic concentrations of IL-6 and IL-8 protein are characteristic of the AAA wall



Aortic wall protein expression levels of IL-6 and IL-8 were determined by specific ELISAs for aneurysmal wall (AAA; n =14) and atherosclerotic wall (ASD; n =12) samples. Results are presented in box plots as the median, lower and upper quartiles and range. *P <0.0001 compared with ASD.



Representative photomicrographs (similar magnification) of ASD (left) and AAA (right) tissue stained with antibodies specifically detecting IL-6 or IL-8. IL-6 immunoreactivity was associated with plasma cells and macrophages; IL-8 immunoreactivity was associated with lymphocytes and neutrophils based on morphological characteristics. Scale bar, 100 µm.

Examination of the molecular and cellular effects downstream of IL-6

IL-6-mediated responses depend on the availability of cell-bound IL-6R or sIL-6R. sIL-6R can induce IL-6 signaling in cells not constitutively expressing IL-6R (IL-6 trans-signaling) but expressing its receptor dimer gp130[23]. Figure 4(A) shows that sIL-6R was present in the aortic wall, and that levels were comparable in AAA and ASD samples. Quantification of phospho-STAT-3, a downstream transcriptional effector of IL-6, showed that the AAA wall

TABLE 4

ΔCt

Cytokine mRNA	Fold increase in AAA	P value	AAA	ASD
IL-6	29.9 (10.9–86.8)	0.001	2.8 ±1.7	7.7 ±2.0
IL- 8	21.1 (3.48–65.3)	0.003	1.4 ±2.1	5.8 ±4.5

Increase in IL-6 and IL-8 gene expression in the AAA wall

mRNA expression of IL-6 and IL-8 was analysed in aneurysmal wall (AAA; n =17) and atherosclerotic wall (ASD; n =12) samples. The gene expression level of the measured genes in ASD was set at 1, and relative gene expression levels in AAA tissues are expressed as median fold increase, together with the corresponding 95 % CI of the median fold increase and the P value. The mean (±S.D.) ΔCt values are provided in the last two columns. High (low)ΔCt values reflect low (high) mRNA expression levels.

contained higher levels of phospho-STAT- 3 (20-fold increase; $P<0.01$) compared with the ASD wall (Figure 4B). The protein levels of the inactive form (nonphosphorylated STAT-3) were lower in the AAA wall (Figure 4B). Taken together, these results indicate that hyperexpression of IL-6 in AAAs is associated with elevated levels of phospho-STAT3, i.e. enhanced IL-6 signaling.

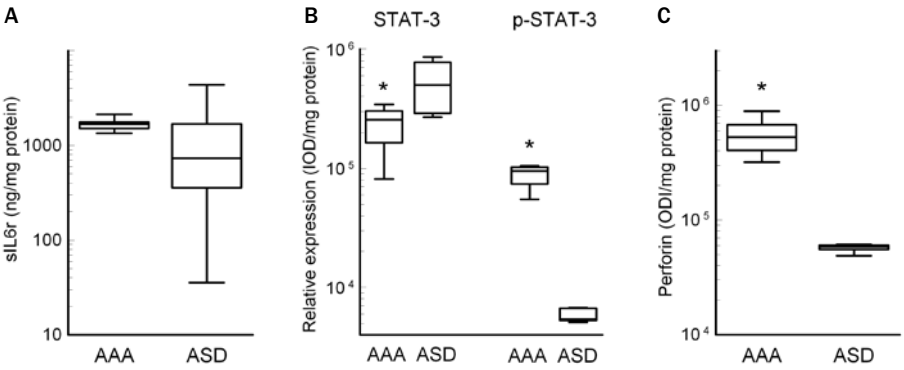
Well-established functional downstream effects (readouts) of IL-6 include the differentiation/ maturation of B-cells into plasma cells and the activation of cytotoxic T-cells. Evaluation of the plasma cell content in AAA and ASD samples using IHC demonstrated an abundance of plasma cells in the AAA wall, whereas plasma-cell specific immunoreactivity (CD138) was not, or hardly, present in the ASD wall (Figures 1C and 1D). Expression of perforin, a factor which indicates cytotoxic T-cell activation, was significantly elevated in AAA samples, both at the transcriptional (13-fold, P=0.006; results not shown) and protein (Figure 4C) expression levels.

Examination of the molecular and cellular effects downstream of IL-8

IL-8 strongly promotes neutrophil infiltration and activation, and can exert strong pro-angiogenic activities. Quantification of infiltrated neutrophils by IHC staining of MPO demonstrated a pronounced recruitment of these cells in AAA samples, but only a scattered presence of neutrophils in the adventitial layer of ASD samples (Figures 1E and 1F).

To examine whether increased pro-angiogenic responses were associated with AAAs, the number of vasa vasorum in AAA and ASD samples were quantified. The doubling of the number of vasa vasorum (9/mm2 in AAA samples compared with 4/mm2 in ASD samples) points to an enhanced angiogenic response in AAAs (results not shown).

FIGURE 4



Assessment of the functional components of the IL-6 signaling pathway in AAA and ASD walls. (A) Aortic wall protein expression levels of sIL-6R was determined by ELISA from aneurysmal wall (AAA; n =14) and atherosclerotic wall (ASD; n =12) samples. (B) Basal levels of STAT-3 protein and levels of the activated form [phospho-STAT-3 (p-STAT-3)] were determined by Western blotting in AAA and ASD homogenates. Expression was determined relative to β-actin. *P <0.0001 compared with ASD. (C). Aortic wall protein expression levels of perforin was determined by ELISA from aneurysmal wall (AAA; n =14) and atherosclerotic wall (ASD; n =12) samples. Elevated perforin protein levels in the aneurysmal wall indicate increased cytotoxic T-cell activation in AAAs. Results are presented in box plots as medians, lower and upper quartiles and ranges. *P <0.001 compared with ASD. IOD (ODI), integrated optical density.

Discussion

In the present study, we set out to determine the aortic levels of inflammatory cytokines in relation to downstream inflammatory transcription factors and cellular responses in human AAAs. Comparison of growing AAAs with ASD using biopsies taken from the same aortic region allowed us to define inflammatory characteristics of AAAs that distinguish the disease from ASD.

The results of the present study demonstrate an enhanced expression of factors associated with both Th1- and Th2-type responses in AAAs. Our findings do not, therefore, support a clear Th1 or Th2 polarization in AAAs. At the transcription factor level, AAAs clearly differ from ASD by having higher concentrations of the pro-inflammatory transcription factors C/EBPα, C/EBPβ and C/EBPδ, and the activated forms of p65 NF-κB and c-Jun, a component of AP-1. Consistent with this, AAAs are characterized by hyperexpression of IL-6 and IL-8, both of which are positively regulated by NF-κB and AP-1. We demonstrate that molecular and cellular processes that are associated with hyperactivation of IL-6 and IL-8 are exaggerated in AAAs: the aortic phospho-STAT-3 and perforin concentrations are elevated in the AAA wall, and B-cells, plasma cells and neutrophils are abundantly present in AAA samples, clearly distinguishing this pathology from ASD.

The large majority of patients with an AAA also suffer from advanced atherosclerotic disease, and AAAs have long been considered to be one of the classical manifestations of atherosclerotic disease. However, traditional atherosclerotic risk factors, such as cholesterol and diabetes, are not associated with an AAA[24], suggesting that an AAA may be

a separate entity. Although AAAs and ASD share an inflammatory component relevant for disease evolution, the exact molecular processes causatively involved in the progression of AAAs or ASD are not fully understood. In addition, clear-cut mechanistic differences allowing the discrimination of AAAs from ASD have not been identified to date.

Recent studies assessing the aortic expression of Th1- and Th2-specific inflammatory mediators in human AAAs report controversial observations and claim a predominance of either Th1 type or Th2-type responses[3,10,11]. Our present findings do not support a clear Th1/Th2 polarization in AAAs and indicate an upregulation of both immune cell responses (as compared with ASD), together with a profound general inflammatory response, which is characterized by the high expression of cytokines, chemokines and growth factors [e.g. MCP-1, MIF, TGF-β (transforming growth factor-β), MIP-1β and G-CSF]. Our present observations are consistent with a recent protein array study by Middleton and co-workers[12], who characterized the AAA as a classical inflammatory condition which is dominated by a generic inflammatory response involving similar pro-inflammatory (e.g. IL-1β and TNF-α) and anti-inflammatory cytokines (e.g. IL-10)[25].

The net effect at the downstream transcription factor level has not been investigated to date. Our present findings demonstrate for the first time that the aortic concentrations of major inflammatory transcription factors at baseline (C/EBPα, C/EBPβ and C/EBP δ) or their activated forms (p65 NF-κBactive and phospho-c-Jun) are significantly increased in AAAs relative to ASD. Elevated aortic levels of phospho-c-Jun are in accordance with an increased activation of JNK (c-Jun N-terminal kinase) in an AAA as reported previously[26]. As IL-8 and IL-6 gene expression depend on simultaneous activation of NF-κB and AP-1[21,22], the aortic concentrations of these cytokines in AAAs and ASD were examined at the gene transcription level. Indeed, IL-6 and IL-8 mRNA were markedly elevated in AAA samples and this was paralleled with a significant increase in IL-6 and IL-8 protein levels. High aortic expression levels of IL-6 and IL-8 in human AAAs have also been reported by others[13,14,27], but the extreme disparity compared with ASD has not been recognized to date. The hyperexpression of IL-6 and IL-8 in the AAA samples observed in the present study thus constitutes an important difference between the pathologies of AAAs and ASD. Predominance of IL 6 and IL-8 in the growing AAA and responses mediated by these cytokines point to a central role of these factors in the development of AAAs.

IL-6 is a well-recognized inducer of the hepatic acute phase response and a possible cardiovascular risk factor[28]. Because the IL-6R is expressed predominantly by hepatocytes and leucocytes, an extrahepatic role of IL-6 was uncertain. With the discovery of sIL-6R allowing ‘IL-6 trans-signaling’ in cells normally not expressing IL-6R[23], IL-6 has gained importance in vascular disease, and in atherosclerosis IL-6 has been associated with lipid homoeostasis, vascular remodeling and plaque remodeling[29]. Our present results demonstrate that sIL-6R is expressed in AAA and ASD walls to a comparable extent. We have also demonstrated that a key mediator and downstream effector of IL-6, STAT-3, is present in the aorta, together with its activated (phosphorylated) form phospho-STAT3.

We have shown that the large differences in IL-6 protein expression in AAA and ASD samples are paralleled at the downstream transcription factor level: AAA walls contain higher phospho-STAT-3 concentrations than ASD, pointing to a pronounced activation of the IL-6 (trans) signaling route in AAAs.

Increased IL-6 (trans) signaling may, at least partly, explain the differences in cellular composition seen in AAA and ASD wall samples. IL-6 reportedly controls late B-cell differentiation and plasma cell formation, and is an established promoter of T-cell migration, retention and activation[30,31]. Enhanced activation of the IL-6 route may very well be responsible for the abundant infiltration of B cells observed in the present study and by others[14,32]. Furthermore, the histological results in the present study indicate a high abundance of plasma cells (plasmacytosis) in the AAA samples, suggesting a higher B-cell differentiation rate[33]. Analysis of CD4 and CD8 expression indicates mainly increased infiltration of cytotoxic T-cells in AAA samples. Moreover, increased perforin expression, an established marker of cytotoxic T-cell activation[34], demonstrated an increased cytotoxic T-cell activation status in AAA compared with ASD samples, which is consistent with the observation that apoptosis is enhanced in AAAs[35].

Increased IL-8 expression has been reported in several inflammatory conditions, including Kawasaki's disease[36] and Behc,et disease[37], two conditions that are associated with aneurysm formation. Consistent with this, expression of IL-8 in aortic aneurysmal tissue has been described[12,27]. To our knowledge, the hyperexpression of IL-8 associated with neutrophil cell infiltration in the AAA samples observed in the present study has not been reported to date and suggests that neutrophil infiltration is a hallmark of an AAA, allowing the discrimination of AAAs and ASD. Furthermore, G-CSF, the simultaneous expression of which facilitates IL-8-dependent Neutrophil cell recruitment[38], was highly expressed in the AAA, but not in ASD, samples. It is tempting to speculate that the concomitant expression of these factors is responsible for neutrophil infiltration which appears critical for the process of aneurysm formation[39,40].

In summary, our present results demonstrate that inflammation in the AAA wall is clearly distinct from inflammation in ASD, and that IL-6 and IL-8 hyperexpression and the dominance of IL 6- and IL-8-mediated responses prevail in an AAA. This comprehensive inflammatory response in an AAA may well be responsible for the broad, albeit not universal, up-regulation of selective members of the MMP (matrix metalloproteinase) class of proteases[2,16] {MMP-8 (neutrophil-derived) and MMP-9 (AP-1-regulated[26])}, and the cysteine proteases cathepsin K, L and S[16] (NF-κB-[41], C/EBP-[42] and IFN-γ -[43] regulated respectively) in the disease. Anti-inflammatory strategies attenuating IL-6 and IL-8 expression or activity[26], and/or IL-6 and IL-8 signaling, may prove effective for the pharmaceutical stabilization of AAAs.

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The pathophysiology of abdominal aortic aneurysm growth: corresponding and discordant inflammatory and proteolytic processes in abdominal aortic and popliteal artery aneurysms.

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Abstract

Introduction: There is remarkable controversy over the processes driving abdominal aneurysm growth. Elucidation of the key inflammatory and proteolytic processes is hampered by the inherent limitations of animal and human studies. Human data is largely derived from surgical specimens which typically reflect the final stages of the disease process and thus do not allow distinction between primary and secondary processes. Clear epidemiological and genetic associations between abdominal and popliteal aneurysms suggest that that these two pathologies share common grounds. On this basis we reasoned that information of corresponding and discordant processes in these aneurysms may provide critical clues on the processes that are crucial respectively dispensable for aneurysm progression.

Methods and Results: mRNA (semi quantitative RealTime PCR) and protein analysis (ELISAs, Muliplex, Western Blotting), and histology were performed on aneurysm wall samples obtained during elective popliteal and abdominal aortic aneurysm repair. Non aneurysmatic aorta tissue from organ donors was included as reference. MessengerRNA and protein analysis showed that both aneurysms are characterized by a marked activation of NF-κB and AP-1 proinflammatory transcription factors, and IL-6 and 8 hyperexpression. Clearly discordant findings were found for other inflammatory markers such as interferon γ, IP-10, TNFα, MCP-1 and MIP1α and β which were all lower in PAA. On cellular level both pathologies exhibited profuse infiltration of macrophages, neutrophils and T-helper cells. Results for the B-, plasma- and cytotoxic T-cells were clearly discordant with minimal infiltration of these cell types in PAA. Evaluation of protease expression and 3 activation showed that both conditions are dominated by increased MMP8 and 9, and cathepsin K, L and S expression and activation.

Discussion: This explorative study characterizes degenerative aneurysmal disease general inflammatory conditions dominated by profound activation of the NF-κB and AP-1 pathways, IL6 and 8 hyperexpression, and neutrophil involvement. Clearly discordant findings for interferon γ, cytotoxic T-cells, B-cells, plasma cells challenge a critical role for these factors in the process of aneurysm growth. Pharmaceutical strategies targeting the generic components in AAA and PAA may prove effective for the stabilization of AAA.

Introduction

An abdominal aortic aneurysm (AAA) is a common, dilating disorder of the aorta and a major cause of death due to rupture.[1,2,3] Despite consensus on the fact that AAA is best described as a chronic inflammatory condition with an accompanying proteolytic imbalance, the exact nature of the inflammatory cascades, and the proteases driving aneurysmal growth remain unresolved. For example, while AAA was initially designated by Schonbeck and coworkers as a Th2-type inflammatory disease,[4] later studies suggest that AAA is better characterized as a Th1 dominated,[5] or alternatively as a general (pro) inflammatory disease.[6] With respect to the proteases involved, attention has been primarily focused on the gelatinase MMP9. Yet, despite ample evidence for a critical role of MMP9 in AAA formation and grow in *animal* models of the disease, the pivotal role of MMP9 in driving *human* AAA growth is debated,[7]

and additional proteases such as MMP8 and the cysteine proteases cathepsin K, L and S have now also been brought forward.[8]

Interpretation of the available data is further complicated by the fact that most data is based on surgical specimens and/or on animal models of the disease. Surgical specimens generally represent the final stages of the disease process, making it difficult to discriminate between the primary and secondary events in the disease process.[9,10] Similarly while animal models may provide valuable clues to the disease process, their relevance for AAA in general remains to be established.[9]

Remarkable associations are found between AAA and popliteal artery aneurysms (PAA), the by far most common form of peripheral aneurysms.[11,12] This is not only reflected by the fact that about 40-50% of patients with a PAA are also diagnosed with an AAA,[13,14] but also by the aggregation of AAA and PAA in relatives of patients with these aneurysms. Altogether, these observations suggest that AAA 5 and PAA share common grounds.[15] We reasoned that identification of putative generic factors in the two conditions may provide clues on the nature of the inflammatory and proteolytic pathways involved in aneurysm growth, and constitutes a first important step to identify efficient therapeutic targets.

Since a methodical comparison of AAA and PAA is missing, we now performed a systematic and comprehensive analysis of inflammatory and proteolytic pathways activated in AAA and PAA tissue in order to identify corresponding (i.e. generally relevant) and discordant (i.e. aneurysm-subtype specific) processes in these two related pathologies.[6]

Materials and methods

Tissues and ethical concerns

All aneurysm samples were obtained during elective aneurysm repair. Reference samples (non aneurysmal abdominal aortic wall samples) were obtained from the Vascular Tissue Bank (Department of Vascular Surgery, Leiden, The Netherlands).

Sample collection and handling was performed in accordance with the guidelines of the Medical and Ethical Committee in Leiden, Netherlands and the code of conduct of the Dutch Federation of Biomedical Scientific Societies. All samples were obtained following consent of the patients. None of the patients in the present study had a history of diabetic or chronic inflammatory disease. AAA samples (anterior lateral aneurysm wall) were obtained from patients with an AAA >5.5 cm undergoing elective open repair (AAA group: n=17; age: 72.4 ± 6.2 years (value is the mean ± S.D.); 14 males/3 females; and AAA diameter, 6.7 ± 1.1 cm). PAA samples were from patients with PAA>2,0 cm16 undergoing elective repair (PAA group: n=12; age: 69.6 ± 12.1 years; 11 males, 1 female).

For comparison of the AAA and PAA wall with the normal aortic wall (non aneurysmal control), we used non-aneurysmal aortic wall patches from kidney donations. The patches were obtained during clinical organ transplantation with grafts derived from cadaveric donors.

The primary cause of the fatal brain injury in of kidney donors was a major head trauma or subarachnoidal bleeding. Only patches displaying advanced atherosclerosis with advanced atherosclerotic lesions (equaling the characteristics of grade IV–VI lesions according to the Stary classification[17] were 7 selected (control group; n=11; age, 55.6 ± 10.2 years; seven males/four females; aortic diameter, <2.0 cm). Of note, all control samples were obtained at the level of the renal artery and during a laparotomy, i.e. from a comparable region and during a similar procedure as the AAA samples.

Tissue procurement: any adhering thrombus was carefully removed and the vessel walls were halved lengthwise. One half was immediately snap-frozen (liquid nitrogen) and stored at -80°C for mRNA (RT-PCR) and protein (Western blot and ELISA) analysis. The other half was fixed in formaldehyde (24 h), decalcified (Kristensens solution, 120 h) and embedded in paraffin for histological analysis.

Real-Time PCR

Tissue samples were pulverized in liquid nitrogen and RNA was isolated and semiquantitative Real-Time LightCycler PCR (RT-PCR) was carried out (Taqman method) as detailed in previous publications.[18] Total RNA extraction was performed using RNAzol (Campro Scientific, Veenendaal, The Netherlands) and glass beads according to the manufacturer's instructions. Copy-DNA was prepared using kit #A3500 (Promega, Leiden, The Netherlands) and quantitative real-time polymerase chain reaction (RT-PCR) analysis was performed for human IL-1α, IL-1β, IL-2, IL-6, IL-8, TNFα, MCP-1, MIP-1β, MIF, TGFβ, CD4, T-Bet, GATA-3, IL-4, IL-10, IL-13, INF γ, CD8, Perforin, Granzyme A, BLIMP-1, MAD4, Immunoglobulin linkerprotein, Immunoglobulin heavy chain, MMP- 25, CD 337, Tryptase, MMP2, MMP3, MMP9, MMP13, TIMP-1, TIMP-2, TIMP-3, Cathepsin K, Cathepsin L, Cathepsin S, Cystatin C, uPA and PAI-1 on the ABI-7700 system (Applied Biosytems, Nieuwerkerk a/d IJssel, The Netherlands) using 8 established primer/probe sets (Assays on Demand, Applied Biosystems) and mastermix (Eurogentec, Seraing, Belgium). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as a reference and for normalization.

Tissue homogenization

Snap-frozen samples were crushed in liquid nitrogen and mixed with lysis buffer (10 mM Tris pH 7.0, 0.1 mM CaCl2, 0.1 M NaCl, 0.25% (v/v) Triton X-100) for protein based assays. After centrifugation at 4°C for 15 minutes at 13,000 rpm, the supernatants were collected and protein contents were measured with Pierce (Rockford, IL, USA) before storage at -80°C until use.

IL-6 and IL-8 ELISA

Vessel wall cytokines were measured in the homogenates by the bio plex panel for multiple cytokines (Bio-Rad Laboratories B.V, Veenendaal, The Netherlands, IL-1α, IL-1β, IL-13, IL-17A, TNFα, IFN-MIP-1α, MIP-1β and G-CSF) or by separate ELISAs (PeliKane compact kit (Sanquin, Amsterdam, The Netherlands) for IL-6 and IL 8. We previously concluded that AAA IL-2, IL-4, IL-5, IL-7, IL-10, IL-12, IL-17A and GM-CSF wall levels are all at or below the detection threshold of the multiplex assay. Hence these cytokines were not included in the analysis.

Western blotting

Quantifiable Western blots were performed following detailed protocols described by Kleemann and colleagues.[19] All assays were performed on the tissue homogenates using antibodies specific for the human forms of p65-NFκB (active form of p65-NFκ ; Chemicon, #MAB3026, Chemicon Europe, Ltd., Chandlers Ford, UK); p65-NFκB 9 (non-active form; sc-8008; Santa Cruz, Heerhugowaard, The Netherlands); c-Jun (sc- 45); phosphor (Ser73)-c-Jun (sc-7981); C/EBPα (sc-9315); C/EBPβ (sc-150); C/EBP δ (sc-636); MMP2 (PC-158, the Bindingsite, Birmingham, UK); MMP8 (MAB3316, Chemicon, Chemicon Europe, Ltd., Chandlers Ford, UK); MMP9 (TNO-BEA-21); Cathepsin K (IM55L, Calbiochem, Breda, The Netherlands); Cathepsin L (AF952, R&D systems, Abingdon, UK); Cathepsin S (sc-6505, Santa Cruz) and α-actin (sc- 1615, Santa Cruz) for normalization. All protease antibodies were specifically chosen to effectively detect both the pro and activated forms of the proteases.[8] All secondary antibodies were obtained from Santa Cruz Biotechnology. Immunoblots were visualized using Super Signal West Dura Extended Duration Substrate (Perbio Science, Etten-Leur, The Netherlands), and a luminescent image workstation (UVP, Cambridge, UK). Immunoblots were quantified using LabWorks 4.6 software.

Immunohistochemistry

Immunohistochemistry was performed using deparaffinized, ethanol rehydrated tissue cross-sections (thickness: 4μm) as reported previously.[20] Sections were incubated overnight with polyclonal antibodies specifically for human myeloperoxidase (DAKO, Heverlee, Belgium), CD4 (clone 1F6, DAKO), CD8 (clone 4B11, Novocastra, Valkenswaard, the Netherlands), CD20 (clone L26, DAKO), CD68 (clone KP6, DAKO) and CD138 (clone B-B4, Serotec, Oxford, UK). Conjugated biotinylated anti-goat or rabbit anti-IgG were used as secondary antibodies. Sections were developed with Nova Red® (Vector Laboratories, Burlingame, CA) and counterstained with Mayer Hematoxylin allowing morphological analysis. Specificity of the antibody staining was confirmed by omitting the primary antibody. Specimens 10 were quantified by counting the number of cells per unit area for at least 20 fields at a 400-fold magnification.

Statistical analysis

Messenger RNA and protein expressions, results of the Western blots and immunohistochemistry were analyzed by Wilcoxon-Mann-Whitney U test to compare the different groups. Statistical significance was accepted if the P-value was < 0.05 Most of the data in this study reflects coherent data that fits in the theoretical inflammatory frame works. As such non-corrected data is provided,[21] yet a Bonferroni correction should be considered when interpreting non coherent data. All analyses were performed using SPSS16.0. 11

Results

Patient characteristics are shown in Table 1. The observed younger age and smaller size of PAA confers with clinical data and reflect the younger peak incident age of PAA22 when compared to AAA and smaller size of the popliteal artery and hence the lower intervention threshold for PAA (PAA 2.0 cm, AAA 5.5 cm).

TABLE 1

Patient characteristics (mean ± sd)

	AAA	PAA	Control aorta
Evaluable patients (n)	15	12	11
Mean Age (years)	74.8 ± 6.2	69.6 ± 12.1	55.6 ± 10.2
Mean diameter (cm)	6.7 ± 1.1	3.35 ± 1.36	<2.0
Female sex (n)	1	1	4
Aneurysm elswhere	1	4	0
Current smoker (n)	6	4	4
Statin use (n)	1	3	0
Anti-hypertensives (n)	8	5	0
Anti platelet therapy (n)	10	9	0

Cellular composition and cellular markers in AAA and PAA walls

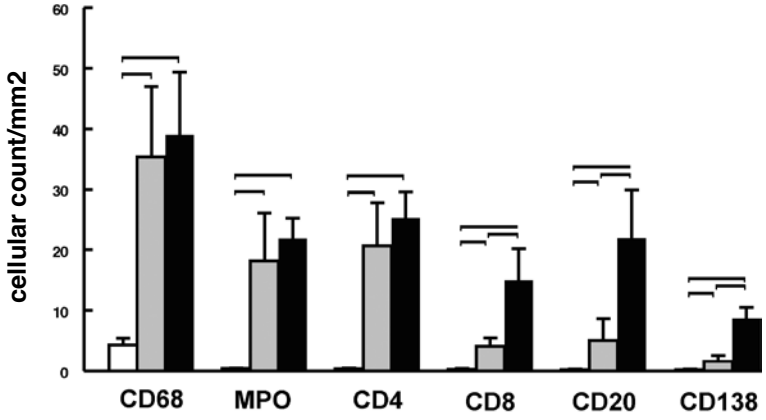
We first performed a histological evaluation of infiltrating leucocytes in AAA, PAA and non-aneurysmal control aorta. Results of these analyses are shown in Figure 1. Both AAA and PAA tissues were characterized by profuse and diffuse infiltration of monocytes (CD68+), neutrophils (MPO+) and T-helper cells (CD4+). Findings for B-cells (CD20+), plasma cells (CD138+) and cytotoxic T-cells (CD8+) content on the other hand were clearly discordant between PAA and AAA, with CD20+, CD138+ and CD8+ abundance in AAA but very limited presence in PAA. The findings for the reduced B-cell, plasma cell and cytotoxic T-cell content in PAA were in accordance with a reduced mRNA expression of B-cell, plasma cell and cytotoxic T-cell markers in PAA (Table 2). In line with the cellular observations no difference was found for the expression of monocyte/macrophage, neutrophil and the T-helper cell markers in AAA and PAA (Table 2).

Analysis of inflammatory molecular factors in AAA and PAA walls

Comparison of AAA and PAA inflammatory fingerprint on the mRNA level (semi quantitative real-time PCR) and protein level shows that PAA and AAA are characterized by particularly prominent IL-6 and 8 expression (Table 2, Figure 2)[12] although the increase in IL-6 levels was less outspoken in PAA than in AAA (P=0.038) (Figure 2). Findings for the other proinflammatory factors such as TNFα, interferon γ and IP-10, MCP-1 and MIPα and β were clearly discordant between AAA and PAA (P<0.008, Table 3).

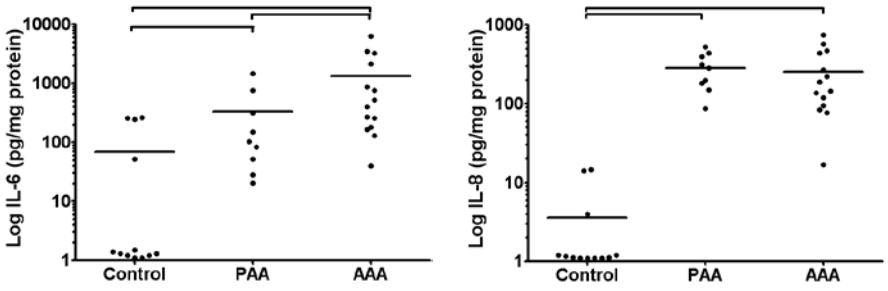
The congruent IL-8 hyperexpression in AAA and PAA prompted us to quantify the baseline levels of the general proinflammatory transcription factors NF-κB, AP-1 and C/EBP. Similar baseline but clearly increased NF-κB and AP-1 activation (active confirmation (NF-κB) and phosphor-c-jun (AP-1)) show that both AAA and PAA are associated with a hyperactivation of these systems6,[23] (Figure 3). Abundance of the C/EBP isoforms α/β/δ was less outspoken in PAA (P<0.021) (Figure 3).

FIGURE 1



Immunohistochemical analysis of the cellular composition of AAA, PAA and control aorta tissue. The amount of cells/mm2 in AAA and PAA tissues, and control aorta is shown after staining with antibodies specifically detecting monocytes and macrophages (CD68), neutrophils (MPO), T helper cells (CD4), cytotoxic T cells (CD8), B-lymphocytes (CD20) and plasma cells (CD138). There is no significant difference between AAA and PAA with respect to CD68, MPO and CD4 . Significant differences were found in the CD8, CD20 and CD138 content. *P <0.05. White = control aorta, grey = PAA, black = AAA

FIGURE 2



Concentrations of IL-6 and IL-8 protein are characteristic for AAA and PAA wall. Aortic wall protein expression levels of IL-6 and IL-8 were determined by specific ELISAs in PAA and AAA wall and control aorta samples. There was strong significant difference between the control aorta and the aneurysm group with respect to IL-6 and IL-8. Focussing on AAA and PAA, IL-8 hyperexpression was found in the two conditions but IL-6 hyperexpression was less outspoken in PAA. *P <0.05.

TABLE 2

		PAA	AAA	*p*-value PAA vs AAA	Non-aneurysmal Aortic Control	*p*-value PAA vs Control
	IL-1 α	-3,12 [-3,32 – -2,81]	-2,49 [-3,02 – -1,80]	0,065	ND	0.071
	IL-1 β	-1,40 [-2,03 – -1,31]	-0,80 [-1,17 – -0,53]	0,007*	-1,91 [-2,74 – -1,35]	0.270
	IL-2	-3,15 [-4,83 – -2,91]	-2,92 [-3,25 – -2,34]	0,135	-4,86 [ND – -3,60]	0.091
	IL-6	-0,76 [-0,84 – -0,73]	-0,77 [-1,07 – -0,45]	0,604	-2,19 [-2,81 – -1,84]	0.00013
	IL-8	-0,56 [-1,28 – -0,14]	-0,24 [-0,84 – 0,02]	0,348	-1,53 [-2,61 – -0,86]	0.085
Cytokines	TNFα	-1,95 [-2,72 – -1,74]	-2,43 [-2,65 – -1,92]	0,452	-2,81 [-3,80 – -1,99]	0.188
	MCP-1	0,11 [ND – 0,64]	0,54 [0,10 – 0,92]	0,26	0,11 [-0,41 – 0,47]	0.758
	MIP-1β	-0,71 [-0,84 – -0,72]	-0,40 [ND – -0,11]	0,008*	-1,05 [-1,58 – -0,44]	0.230
	MIF	0,49 [0,32 – 0,59]	0,56 [0,41 – 0,83]	0,222	0,28 [0,20 – 0,37]	0.246
	TGFβ	-0,18 [-0,49 – 0,12]	0,30 [0,04 – 0,48]	0,001*	0,14 [-0,05 – 0,34]	0.069
	CD4	-2,75 [-2,89 – -2,12]	-2,38 [-2,74 – -2,26]	1	-3,46 [-4,22 – -2,12]	0.328
	T-Bet	-2,67 [-3,44 – -2,29]	-2,64 [-4,96 – -1,81]	0,72	-4,01 [ND – -3,25]	0.069
	GATA-3	-3,04 [-3,38 – -2,92]	-2,04 [-2,64 – -1,46]	0,001*	-3,13 [ND – -2,35]	0.930
T-helper cell	IL-4	ND	-3,59 [-4,11 – -3,38]	0,010*	-3,83 [-4,62 – -3,46]	0.126
	IL-10	-2,09 [-2,50 – -1,92]	-1,74 [-2,22 – -1,35]	0,106	-1,89 [-2,28 – -1,35]	0.525
	IL-13	ND	-3,29 [-4,88 – -2,47]	0,166	ND	0.375
	Interferon γ	-4,97 [ND – -2,92]	-2,71 [-3,05 – -2,23]	0,008*	-5,52 [ND – -3,04]	0.536
	CD8	-2,81 [-3,12 – -2,10]	-1,49 [-1,75 – -1,06]	0,001*	-2,91 [-4,30 – -1,89]	0.596
Cytotoxic T cell	Perforin	-2,85 [-3,31 – -2,72]	-2,25 [-2,76 – -1,53]	0,034*	-3,38 [-4,07 – -2,88]	0.230
	Granzyme A	-1,67 [-1,92 – -1,41]	-0,83 [-1,13 – -0,50]	0,003*	-1,96 [-2,41 – -1,04]	0.179
	BLIMP-1	-2,25 [-2,82 – -2,04]	-1,39 [-2,05 – -1,25]	0,023*	-2,92 [-3,47 – -2,48]	0.081
B/Plasma Cell	MAD4	-1,45 [-1,63 – -1,31]	-1,05 [-1,49 – -0,83]	0,044*	-1,41 [-1,56 – -1,05]	0.808
	Iglinkerprotein	-0,40 [-0,92 – -0,44]	0,26 [-0,02 – 0,63]	0,001*	-1,21 [-1,70 – -0,73]	0.122
	Ig heavy chain	0,17 [-0,44 – 0,74]	1,76 [1,29 – 2,10]	0,001*	0,18 [-1,18 – 0,56]	0.536
Neutrophil	MMP-25	-1,59 [-1,91 – -0,92]	-1,80 [-2,01 – -1,12]	0,757	-2,94 [-3,52 – -2,73]	0.027
NK cell	CD 337	-3,35 [-3,79 – -2,77]	-2,22 [-2,75 – -1,39]	0,006*	-4,16 [-4,99 – -3,12]	0.180
	MMP2	-1,02 [-1,35 – -0,96]	-2,64 [-3,31 – -2,13]	0,001*	-2,55 [-3,09 – -1,60]	0.015
	MMP3	-2,21 [-2,87 – -1,35]	-2,42 [-3,17 – -2,05]	0,349	-3,38 [-4,53 – -2,61]	0.027
	MMP9	-1,06 [-1,26 – -0,82]	-1,27 [-1,56 – -0,87]	0,288	-2,54 [-3,34 – -1,24]	0.011
	MMP13	-2,11 [-2,36 – -1,25]	-3,15 [-3,51 – -2,74]	0,018*	-3,46 [-3,66 – -2,66]	0.043
	Cathepsin K	-1,79 [-2,71 – -1,69]	-1,83 [-2,20 – -1,37]	0,376	-2,30 [-2,95 – -1,87]	0.375
Proteases	Cathepsin L	0,67 [0,22 – 0,81]	0,18 [-0,16 – 1,05]	0,619	-0,14 [-0,62 – 0,55]	0.020
	Cathepsin S	-0,52 [-0,87 – -0,48]	-0,71 [-0,84 – -0,40]	0,619	-1,19 [-2,13 – -0,32]	0.211
	TIMP-1	0,74 [0,01 – 0,87]	0,63 [0,22 – 1,26]	0,418	0,25 [0,03 – 0,85]	0.536
	TIMP-2	-1,50 [-1,72 – -1,36]	-1,37 [-1,54 – -0,93]	0,178	-1,78 [-2,03 – -1,33]	0.216
	TIMP-3	-0,54 [-0,81 – -0,35]	-0,54 [-0,90 – 0,17]	0,973	-0,45 [-0,62 – -0,17]	0.301
	Cystatin C	0,72 [0,32 – 0,91]	0,93 [0,69 – 1,20]	0,087	0,87 [0,75 – 1,01]	0.270

Log relative mRNA expression (expression normalized on basis of GAPDH expression) of markers of inflammatory responses in AAA, PAA and control aorta. The numbers are the median [IQR]. P value is for the comparison of AAA versus PAA.

Protease profiles in AAA and PAA

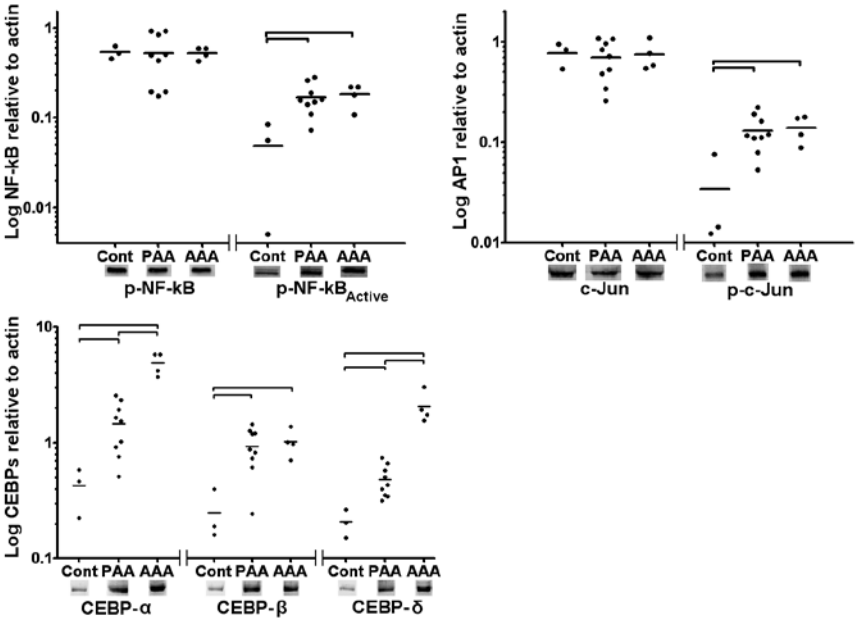
MMP2, 8, 9 and cathepsin K, L and S have been implicated as the primary proteolytic culprits in AAA.[8] Evaluation of these proteases on the messenger and protein level showed a similar profile in PAA (Table 2) , although a trend towards lower expression of the cysteine proteases was observed in PAA. Activation of these proteases was assessed by quantification of the activated forms in Western blot analysis.[8] With the sole exception of reduced cathepsin K and increased MMP2 activation in PAA (p=0.005), similar activation profiles were found in AAA and PAA (Figure 4).[13]

TABLE 3

	AAA	PAA	P
IL-1α	ND [ND – 0.14]	0.14 [ND – 0.34]	0.131
IL1β	1.50 [0.87 – 2.46]	2.81 [2.00 – 7.54]	0.04
IL13	ND [ND – 0.06]	0.18 [0.09 –0.28]	0.001
TNFα	ND [ND – 0.01]	0.04 [0.01 – 0.11]	0.007
Interferon γ	ND [ND – ND]	1.32 [ND – 3.62]	0.008
IP-10	5.7 [1.3 – 38.8]	141.2 [63.0 – 237.0]	0.0001
MCP-1	67.3 [14.2-127.3]	197.2 [102.1 – 212.7]	0.006
MIP-1α	1.29 [0.38 – 1.83]	4.65 [3.52 – 9.81]	0.0003
MIP-1β	2.77 [0.55 – 5.71]	20.0 [10.1 – 26.6]	0.001
G-CSF	ND [ND – 0.13	0.73 [0.40 – 1.77]	0.001

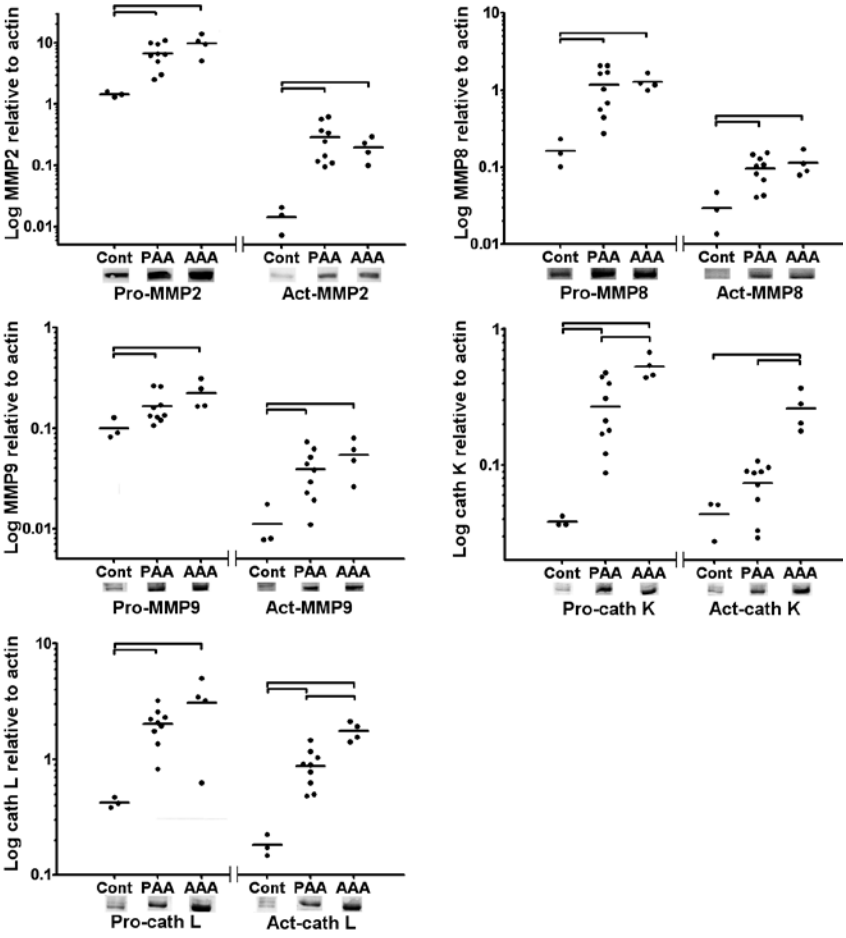
Aortic wall cytokine protein levels normalized on basis of protein levels (pg/mg protein). The numbers are the median [Inter Quartile Range]

FIGURE 3



Concentrations of inflammatory transcription factors are characteristic for AAA and PAA wall. Relative (normalized on basis of β-actin levels) basal levels of p65-NF-κB, c-jun protein and their activated forms (NF-κBactive and p-c-jun) and C/EBPα, C/EBPβ, and C/EBPδ in homogenates of AAA, PAA and control aorta. NF-κB and AP-1 baseline and activation levels were comparable in AAA and PAA. The three isoforms C/EBPα, C/EBPβ, C/EBPδ were significantly lower in PAA. *P <0.05.

FIGURE 4



Pro and active forms of MMP and cathepsin proteases are characterized for AAA and PAA. Westernblot analysis of pro and activated forms of MMP2, MMP8, MMP9, cathepsin K, L and S in AAA, PAA and aorta control tissues. Equal dominance of MMP2, MMP8 and MMP9 were found in AAA and PAA both for the pro and active (act) form. Results for the cathepsins were more variable and less outspoken in PAA when compared to AAA *P < 0.05.

Discussion

The primary processes driving AAA growth are still unresolved. To obtain clues on the factors that are generic for aneurysm growth we performed a systematic comparison of inflammatory and proteolytic processes in AAA and PAA. Our findings demonstrate that both pathologies share a general pro-inflammatory profile that is dominated by IL-8 and IL-6 hyperexpression. Abundance of neutrophils, macrophages, and T-helper cell infiltration, as well as ample expression of MMP and cysteine proteases are clear features of the diseases.

Distinct differences were found for the presence of cytotoxic T-cells, and B- and plasma cells and their associated markers, suggesting that abundance of these cells in AAA is not generic to the process of aneurysmal growth.

The pathology of AAA is complex and poorly understood.[1] Remarkable controversy exists on the inflammatory cascades driving aneurysm growth.[24] In a first evaluation, Schonbeck and colleagues reported dominance of the Th2 associated cytokines IL-4, IL-5 and IL-10, along with minimal expression of the Th1 associated cytokine interferon-γ in AAA.[4] On this basis the authors concluded that AAA is best described as a Th2 driven disease. As opposed to these findings, Xiong et al (mouse model) and Galle et al (human AAA) reported a Th1 polarization in AAA.[5,25] As of these apparent contrasting findings we performed a comprehensive evaluation of the inflammatory fingerprint of AAA and concluded that in AAA is best described as a general pro-inflammatory condition with IL-6 and IL-8 hyperexpression, and dominance of IL-6 and IL-8-related responses.[6] The persistent inflammatory state in AAA is held responsible for a proteolytic imbalance that underlies the excess matrix degradation in the disease. With respect 14 to the proteases involved attention has been primarily focused on the gelatinases MMP2 and 9.[26,27] Yet, it has been pointed out that these proteases cannot degrade the load bearing fibrillar collagens in the aortic wall,[7,8] and that it is thus unlikely that these proteases are directly responsible for the weakening of the aortic wall. As such it is very likely that other proteases contribute to the weakening of the aortic wall as well. As of the large number of open questions and the complexity of the inflammatory and proteolytic cascades in AAA we reasoned that a systematic evaluation of common and discordant inflammatory and proteolytic processes in AAA and PAA may provide critical clues on key processes driving aneurysm growth.

Evaluation of the inflammatory and proteolytic fingerprints in AAA and PAA shows remarkable similarities and dissimilarities. Both conditions are characterized by a strong activation of the general inflammatory transcription factors NFκB and AP- 1 (c-jun), profuse IL-8 and IL-6 expression, a high neutrophil and macrophage content, and abundant expression and activation of the MMP proteases neutrophil collagenase (MMP8) and gelatinase (MMP9) and the cysteine proteases cathepsin K, L and S. As opposed to the congruent findings for IL-6 and 8 much lower levels were found for soluble factors such as interferon γ, the interferon γ-induced protein (IP-10, CXCL-10), TNF α, MCP-1, MIP-1α and β in PAA. These latter observations may indicate that these factors are less critical to aneurysm growth and particularly challenge a role for interferon γ in the progression of AAA.[28] Dominance of neutrophil-derived proteases and neutrophil abundance in AAA and PAA biopsies implies that these cells are generic for these forms of aneurysmal disease. Neutrophils have long been considered part of an acute inflammatory response and their role in chronic conditions has long been ignored.[29] Recent studies[15] ,however show that neutrophils do participate in chronic inflammatory processes such as COPD and rosacea.[29,30] We previously identified the neutrophil derived collagenase MMP8 as the most prominent MMP protease in AAA and as an important target for doxycycline therapy in AAA,[8,31] suggesting that neutrophils may also be actively involved in AAA growth. This notion is support by animal studies by Eliason[32]

and Pagano[33] that both show that abrogated neutrophil influx in an animal models of AAA inhibits AAA development. Neutrophil abundance in AAA and PAA can well be explained by the local IL-8 hyperexpression that is found in the two pathologies. This notion is supported by observations from a clinical trial showing a strong association (r=0.84) between aortic wall neutrophil content and IL-8 levels.[31]

Remarkable differences were found with respect to the cytotoxic T-cell, B-cell and plasma cell content between AAA (abundant) and PAA (minimal). These differences on the histological levels were confirmed by a clear reduction of cell-type specific markers on the messenger RNA level. A clear cut explanation for this finding is missing but possible explanations are the reduced expression of C/EBP family of transcription factors in PAA[34,35] or alternatively that the cytotoxic T-cell, plasma cell and B-cell abundance in AAA reflects presence of the vascular associated lymphoid tissue[36] (VALT) in this section of infra renal aorta.

One possibility is that the reduced cytotoxic T-cell content reflects skewing of the Th1/Th2 balance towards a more Th1 dominated response as indicated by similar T-bet but lower GATA-3 levels in PAA. Yet, such a scenario is not supported by the reduced interferon γ and IP-10 levels in the disease. Another possibility is that the differences in the T-helper cell and cytotoxic T-cell content relates to diverging roles of specific signaling pathways in CD8+ T cell and CD4+ T cell biology.[37,38] Minimal B16 cell and plasma cell infiltration in PAA challenges a critical role for these cells (and the concept of AAA as an auto-immune phenomenon) in aneurysm growth. Yet, little is known about the pathophysiological role of these cells in the context of AAA and further mechanistic studies are necessary to investigate the contribution of these cells and the relevance of a possible auto immune phenomenon[39,40] to AAA formation and progression. The proteolytic imbalance in AAA with prominent expression of MMP2, 8, 9 and the cysteine proteases cathepsin K, L and S is a key factor of AAA. With the notable exception of reduced cathepsin K activation and increased MMP2 in PAA, the inflammatory differences between AAA and PAA were not followed by a change in MMP or cysteine protease levels or activation. We have no clear explanation for the reduced cathepsin K and increase MMP2 activation in PAA. One possible explanation for the reduced Cathepsin K activation in PAA is that it reflects reduced expression and/or activity of the osteoclastic proton pump V-H-ATPase[8] that is required for maintenance of an acidic pericellular micro environment that is required for cathepsin K activation and stability.

In conclusion, our explorative study demonstrates that growing AAA and PAA are both best described as a localized general inflammatory condition that is dominated by activation of NF-κB and AP-1 pro-inflammatory transcription factors and IL-6 and 8 hyperexpression. Dominance of neutrophil and macrophage derived proteases point to a generic role of these cell types in AAA and PAA progression, whereas the discordant findings for B cells, plasma cells and cytotoxic T cells challenge a critical role for these cell types in aneurysm progression. Therapies[17] targeting neutrophil influx or macrophage activation appear the preferred strategy for stabilizing growing AAA.

Our findings demonstrate remarkable overlap between AAA and PAA, and characterize the two pathologies as general inflammatory conditions dominated by activation of the NF-κB and AP-1 pathways; IL8 hyperexpression and neutrophil involvement, and ample activation of selected MMP and cysteine proteases. Remarkable and consistent differences were found with respect to B-cells, plasma cells and cytotoxic T-cells and their markers, suggesting that these cell types are specific for AAA and not critical to the process of aneurysmal growth. Pharmaceutical strategies targeting the generic components in AAA and PAA may prove effective for the stabilization of AAA.[10 18]

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Clinical trial of doxycycline for matrix metalloproteinases-9 inhibition in patients with an abdominal aneurysm: doxycycline selectively depletes aortic wall neurtrophils and cytotoxic T cells

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Abstract

Background: Doxycycline has been shown to effectively inhibit aneurysm formation in animal models of abdominal aortic aneurysm. Although this effect is ascribed to matrix metalloproteinase-9 inhibition, such an effect is unclear in human studies. We reevaluated the effect of doxycycline on aortic wall protease content in a clinical trial and found that doxycycline selectively reduces neutrophil-derived proteases. We thus hypothesized that doxycycline acts through an effect on vascular inflammation.

Methods and Results: Sixty patients scheduled for elective open aneurysmal repair were randomly assigned to 2 weeks of low-, medium-, or high-dose doxycycline (50, 100, or 300 mg/d, respectively) or no medication (control group). Aortic wall samples were collected at the time of operation, and the effect of doxycycline treatment on vascular inflammation was evaluated. Independently of its dose, doxycycline treatment resulted in a profound but selective suppression of aortic wall inflammation as reflected by a selective 72% reduction of the aortic wall neutrophils and a 95% reduction of the aortic wall cytotoxic T-cell content (median values; $P<0.00003$). Evaluation of major inflammatory pathways suggested that doxycycline treatment specifically quenched AP-1 and C/EBP proinflammatory transcription pathways ($P<0.0158$, NS) and reduced vascular interleukin-6 ($P<0.00115$), interleukin-8 ($P<0.00246$, NS), interleukin-13 ($P<0.0184$, NS), and granulocyte colony-stimulating factor ($P<0.031$, NS) protein levels. Doxycycline was well tolerated; there were no adverse effects. Conclusions: A brief period of doxycycline treatment has a profound but selective effect on vascular inflammation and reduces aortic wall neutrophil and cytotoxic T-cell content. Results of this study are relevant for pharmaceutical stabilization of the abdominal aneurysm and possibly for other inflammatory conditions that involve neutrophils and/or cytotoxic T cells.

Clinical perspective: Pharmaceutical stabilization of abdominal aneurysms, thereby reducing the need for aneurysm repair, holds many promises. Matrix metalloproteinase-9 is considered pivotal to the process of aneurysm formation. Because of its ability to reduce matrix metalloproteinase-9 expression and activity, the tetracycline analogue doxycycline has been brought forward as a promising lead candidate. Although animal studies have convincingly shown that doxycycline inhibits both aneurysm formation and growth, the effects of doxycycline on matrix metalloproteinase-9 are controversial in human studies. In this study, we confirm that doxycycline lowers aneurysmal wall matrix metalloproteinase-9 protein levels but also show that this reduction may be secondary and related to an effect on neutrophils, a cell type loaded with preformed matrix metalloproteinase-9 protein. We demonstrate that doxycycline has a profound but selective suppressive effect on inflammation in abdominal aortic aneurysms; doxycycline specifically lowers aortic wall neutrophil (-76%) and cytotoxic T-cell (-96%) content, 2 types considered crucial for aneurysm formation. Moreover, it is shown that treatment reduces interleukin-6 and -8 hyperexpression, a key feature of abdominal aortic aneurysm. Results of this study are relevant for pharmaceutical stabilization of the abdominal aneurysm and possibly for other (vascular) inflammatory conditions involving neutrophils and/or cytotoxic T cells such as Kawasaki disease and Behçet syndrome.

Introduction

Pharmaceutical strategies inhibiting aneurysm growth, thereby reducing the need for surgical repair, could have major advantages for patients and socioeconomically.[1,2] An abundance of matrix metalloproteinase-9 (MMP9) in growing abdominal aortic aneurysms (AAAs),[3] along with the observation that disruption of MMP9 gene prevents AAA formation,[4] led to the notion that MMP9 is critically involved in AAA formation.[5] It was thus proposed that pharmaceutical inhibition of MMP9 could restore the balance between matrix degradation and deposition,[5] thereby reducing aneurysmal growth.[6]

Independently of its antibiotic properties, the tetracycline analogue doxycycline has been shown to reduce both MMP9 expression and activity,[7] suggesting that doxycycline treatment may reduce aneurysmal growth. Indeed, doxycycline has been convincingly shown to prevent AAA formation in a variety of animal models,[6,8–10] and the results from 2 small clinical studies suggest that doxycycline also reduces AAA expansion in humans.[11,12] Remarkably, although the rationale behind doxycycline therapy is based on its putative effects on MMP9 expression and activity,[6] the effects of doxycycline on MMPs in the human aneurysm are unclear, with published studies[13,14] suggesting that doxycycline acts through a different mechanism.[1]

In vitro studies characterize doxycycline as a pleiotropic anti-inflammatory and immunomodulatory agent.[7] However, the relevance of this well-tolerated compound[15] for modulating inflammation in human disease in general and AAA in particular is unclear. In this prospective clinical trial, we systematically examined the effect of 3 pharmacologically relevant doses (low [50/d], regular [100 mg/d], or high [300 mg/d]) of doxycycline on MMP9 expression and inflammatory processes in the aneurysmal wall of patients scheduled for elective open aneurysm repair. Evaluation of the effect of doxycycline treatment showed that MMP9 was reduced on the protein but not on the mRNA level. Because aortic neutrophils carry large amounts of preformed MMP9 protein and lack de novo protein synthesis,[16,17] we hypothesized that the observed reduction of MMP9 protein may relate to a change in the aortic content of neutrophils. The present study shows that a brief period of 2 weeks of doxycycline treatment significantly reduces the aortic content of neutrophils and of cytotoxic T cells, another cell type considered crucial for the process of AAA formation.[18–21] Based on these findings on the cellular level, a molecular analysis on the level of relevant cytokines and transcription factors[22] was performed next.

Materials and methods

The present randomized, dose-ranging study was performed in patients awaiting open aneurysm repair. The trial included 3 treatment groups and 1 nontreated (control) group. Randomization was performed by block randomization (blocks of 20 patients). Randomization lists were generated by the Leiden University Medical Center Department of Medical Statistics, and randomization was performed by the Leiden University Medical Center pharmacy. The study was performed in an investigator-blinded fashion. Planned open aneurysmal repair

of an infrarenal aortic aneurysm was the primary inclusion criterion. Decision for open repair was based on anatomic (eg, neck, elongation) and patient (age) characteristics and patient preferences. Patients with kidney dysfunction (estimated clearance <30 mL/min), chronic inflammatory disease, or (suspected) so called inflammatory aortic aneurysms were excluded from participation in the study. Before randomization, 2 patients were excluded because of a suspected inflammatory aneurysm. Between November 2001 and April 2005, a total of 60 patients from 4 centers in the Netherlands were randomized to receive doxycycline (at a dose of 50, 100 or 300 mg once daily) or no medication (control group) in the 2 weeks preceding open elective aneurysm repair (AAA diameter >5.5 cm). In 2 patients in the low-dose (50 mg/d) doxycycline group, the operation had to be postponed for logistical reasons; hence, 13 patients were therefore evaluated in this group. Medication was started 14 days before the planned operation, and the last dose was taken in the evening before surgery. Patient compliance was assessed by counting the medication (pills) at the end of the treatment period. This multicenter study was first approved by the Leiden University Medical Center Institutional Review Board; subsequent approval was obtained from the medical ethics committees of the other participating centers as required by Dutch law. Informed consent was obtained from each patient.

Immediately after opening of the aneurysm sac and removal of the adhering thrombus, an aortic wall tissue sample was obtained from the anterior-lateral aneurysm wall at the maximum diameter of the aneurysm. Wall samples were immediately halved; one half was snap-frozen in CO2-cooled isopentane or liquid nitrogen and stored at -80°C for later analysis. The other half was fixed in formaldehyde (24 hours), decalcified (Kristensen's solution, 120 hours), and paraffin embedded for histological analysis. All analyses were performed in an investigator-blinded fashion.

RNA Extraction and mRNA Analysis

Total RNA extraction was performed with RNAzol (Campro Scientific, Veenendaal, the Netherlands) and glass beads according to the manufacturer's instructions. Copy DNA was prepared with the A3500 kit (Promega, Leiden, the Netherlands), and quantitative real-time polymerase chain reaction analysis was performed for MMP9, MMP12, cathepsin K, BLIMP-1, MAD-4, Ig linker protein, and IgG heavy chain on the ABI-7700 system (Applied Biosytems, Nieuwerkerk a/d IJssel, the Netherlands) with established primer/ probe sets (Assays on Demand, Applied Biosystems) and Mastermix (Eurogentec, Seraing, Belgium). Analyses were performed according to the manufacturer's instructions and as previously reported.[23] GAPDH expression was used as a reference and for normalization.

Tissue Homogenization for Protein Analysis

Aortic wall tissues were pulverized in liquid nitrogen and homogenized in 2 volumes of lysis buffer (10 mmol/L Tris, pH 7.0; 0.1 mmol/L CaCl2; 0.1 mol/L NaCl; 0.25% [vol/vol] Triton X-100). This protocol releases both soluble and membrane-bound proteins. Samples were subsequently centrifuged at 10 000g for 15 minutes at 4°C, snap-frozen in liquid nitrogen, and stored at -80°C until use. Protein content in homogenates was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill).

Immunohistochemistry

Immunohistochemistry was performed with deparaffinized, ethanolrehydrated tissue cross sections (thickness, 4 μm) as reported previously.[23] Cross sections were incubated overnight with polyclonal antibodies specifically staining human myeloperoxidase (rabbit polyclonal; 1:4000 dilution; DAKO, Heverlee, Belgium), CD4 (clone 1F6; 1:15 dilution; DAKO), CD8 (clone 4B11; 1:200 dilution; Novocastra, Newcastle, UK), CD20 (clone L26; 1:1000 dilution; DAKO), CD68 (clone KP6; 1:400; DAKO), and CD138 (clone B-B4; 1:1000 dilution; Serotec, Oxford, UK). Conjugated biotinylated anti-goat or rabbit anti-IgG was used as a secondary antibody. Sections were developed with Nova Red (Vector Laboratories, Burlingame, Calif) and counterstained with Mayer hematoxylin, allowing morphological analysis. Specificity of the antibody staining was confirmed by omitting the primary antibody and by isotype controls.

Specific Immunocapture MMP Activity Assays

Because of the rapid inactivation of active enzymes by endogenous inhibitors released after cell destruction by homogenation, direct measurement of active MMPs in aortic tissue homogenates is not feasible.[23] Therefore, MMP activities were measured only after in vitro activation (p-aminophenylmercuric acetate) of captured latent proenzymes. Activity was determined in established MMP8 and MMP9 immunocapture protease activity assays (Amersham Biosciences, Buckinghamshire, UK).

Western Blot Analysis

Western blot analyses was performed essentially as described previously[22] with antibodies specific for the human forms of perforin (sc-7417; Santa Cruz Biotechnology, Heerhugowaard, the Netherlands), granzyme A (M1791; Sanquin, Amsterdam, the Netherlands), p65-nuclear factor (NF)κB (nonactive conformation; sc-8008), p65- NFκB (active conformation of p65-NFκB; Chemicon, Billerica, Mass; MAB3026), c-Jun (sc-45), phospho(Ser73)-c-Jun (sc-7981), C/EBPα (sc-9315), C/EBPβ (sc-150), C/EBPδ (sc-636), and STAT3 (sc-7179) (all Santa Cruz Biotechnology); phospho-STAT3 (Epitomics PS727, Huissen, the Netherlands); and β-actin (sc-1615; Santa Cruz Biotechnology).

Corresponding secondary antibodies were obtained from Santa Cruz Biotechnology (donkey anti-goat) and Pierce (Etten-Leur, the Netherlands; goat anti-rabbit and goat anti-mouse). Immunoblots were visualized and quantified with the Super Signal West Dura Extended Duration Substrate (Perbio Science, Etten-Leur, the Netherlands), LabWorks 4.6 software, and a luminescent image workstation (UVP, Cambridge, UK). Protein expression levels were standardized for β-actin, and separate anti–β-actin immunoblots were performed for each sample.

Multiplex Assay and ELISAs

Aneurysm wall interleukin (IL)-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage–CSF, interferon-γ, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1β, and

tumor necrosis factor-α protein levels were determined by the 17-Plex panel for multiple cytokines (Bio-Rad Laboratories BV, Veenendaal, the Netherlands). All analyses were performed in a single run. Detection thresholds for the above cytokines were <0.5 pg/mL (IL-1, IL-2, IL-5, IL-6, IL-7, IL- IL-8, IL-10, tumor necrosis factor-_), <1 pg/mL (IL-12, IL-17A), and <5 pg/mL (IL-4, IL-13, G-CSF, granulocyte macrophage–CSF, interferon-γ, MCP-1).

Most of the IL-6, IL-8, and MCP-1 levels exceeded the upper detection limit of the 17-Plex panel and were reevaluated in separate ELISAs (IL-6 and IL-8: PeliKane Compact Kit, Sanquin, Amsterdam, the Netherlands; MCP-1: Quantikine Kit, R&D Systems, Abingdon, UK).

Statistical Analysis

Power calculation for this study was based on data published in Reference 13. In that publication, doxycycline therapy reduced relative MMP9 mRNA expression from 7.2 (SE, 3.1) to 1.3 (SE, 0.5) units. The present study was designed to have a 90% power to detect a 50% reduction of MMP9 mRNA levels at a significance level of 0.05.

All values are expressed as means (SD) for normally distributed data or medians [interquartile range] for nonnormally distributed data (skewed). The level of statistical significance was dictated by Bonferroni-Holm's correction (33 variables). One-way ANOVA for normally distributed data or the Kruskal–Wallis test for nonnormally distributed data did not indicate a difference between the 3 doxycycline doses for all variables studied (all $P>0.16$; detailed data available from the corresponding author on request). To increase the power of the study, all doxycyclinetreated individuals were therefore evaluated as a single group.

The sequence of our analyses was as follows. We first analyzed the effect of doxycycline on MMP9 mRNA and protein levels. Because of the selective reduction of MMP9 protein only, we analyzed putative changes in the aortic wall neutrophil content. On observing an effect on neutrophil content, we performed a further evaluation. This analysis showed that doxycycline treatment selectively reduces aortic wall neutrophil and cytotoxic T-cell content. On the basis of this cellular evaluation, a subsequent analysis of relevant upstream- and downstream-acting cytokines and transcription factors was performed.[20]

Differences between the control group and the doxycycline-treated groups were evaluated in 1-way ANOVA with contrasts (for the normally distributed data) or by the Wilcoxon–Mann–Whitney test in the case of nonnormally distributed continuous data. Possible associations between aortic wall IL-6 and IL-8 levels and cellular content were evaluated by Pearson's correlation. All analyses were performed with SPSS 16.0 (SPSS Inc, Chicago, Ill).

Results

Patients

Three relevant doses of doxycycline (low [50 mg/d], regular [100 mg/d], or high [300 mg/d] were evaluated.24 A total of 60 patients were enrolled in the study. For logistical reasons, aneurysm repair had to be postponed in 2 patients; hence, a total of 58 patients could be analyzed. Doxycycline was well tolerated, and there were no withdrawals.

Patient characteristics are shown in Table 1. All 4 groups are comparable with regard to age, sex, statin use, and AAA diameter.

Statistical analysis did not indicate a difference between the 3 doxycycline doses for all variables studied; hence, all 3 doxycycline doses were combined. Probability values are for the combined doxycycline group versus the control group.

TABLE 1

Patient characteristics

		Doxycycline,mg			
	Control AAA	50	100	300	*P*
Evaluable patients, n	15	13	15	15	NS
Mean age (range), y	74.8 (69-84)	72.7 (62-85)	74.1 (50-88)	72.1 (58-87)	NS
Mean AAA diameter, cm	6.7	6.5	6.3	6.7	NS
Mean time between diagnosis and surgery, mo	6	7	5	5	
Female sex, n	1	2	2	3	NS
Current smoker, n	6	6	7	5	NS
Statin use, n	1	1	1	2	NS
Antihypertensives, n	8	7	8	7	NS
Antiplatelet therapy, n	10	8	8	8	NS

TABLE 2

mRNA expression

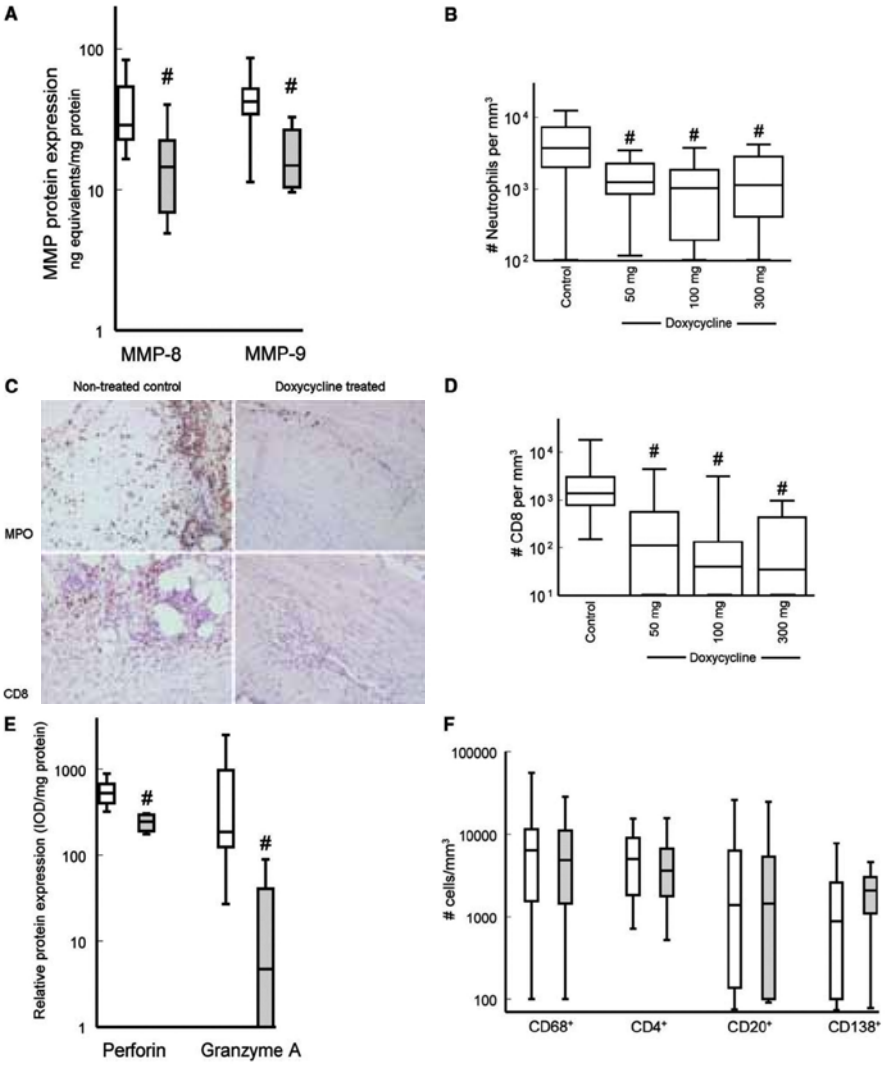
	Nontreated Control Subjects	Doxycycline-Treated Subjects	*P**
MMP9	–1.21 (0.44)	–1.40 (0.61)	0.206 (NS)
MMP12	–2.57 (3.30–1.97)	–2.18 (3.28–1.55)	0.326 (NS)
Cathepsin K	–1.74 (0.69)	–1.85 (0.49)	0.486 (NS)
BLIMP-1	–1.64 (0.59)	–1.81 (0.48)	0.265 (NS)
MAD-4	–1.11 (0.36)	–1.11 (0.25)	0.890 (NS)
Ig linker protein	0.34 (0.39)	0.28 (0.48)	0.714 (NS)
IgG heavy chain	1.73 (0.60)	1.36 (0.48)	0.020 (NS)

NS indicates level of significance not reached after Bonferroni-Holm's correction. Log-transcript level relative to GAPDH (GAPDH=0) of markers of monocyte/macrophage activation (MMP9 and MMP12, cathepsin K) and B-cell/plasma cell activation (BLIMP-1, MAD-4, Ig linkerprotein, IgG heavy chain). Values are mean (SD) or median (interquartile range) as appropriate. *Probability value is for the comparison of the combined doxycycline groups versus the nontreated control group.

Doxycycline and MMP9 Expression

Evaluation of the effect of 2 weeks of doxycycline therapy indicated a trend toward reduced MMP9 proenzyme levels ($P<0.00263$, NS; Figure 1A) but did not influence MMP9 mRNA expression ($P<0.206$; Table 2).

FIGURE 1



The effect of doxycycline on aneurysm wall leukocyte content, the neutrophil markers MMP8 and MMP9, and the cytotoxic T-cell markers perforin and granzyme A. A trend toward reduced MMP9 protein levels (proenzyme) was observed (A) after doxycycline therapy ($P<0.00263$, level of significance not reached after Bonferroni-Holm's correction). Evaluation of a putative effect on cellular content showed that treatment reduced aortic wall neutrophil (B, C; myeloperoxidase staining) and cytotoxic T-cell (C, D; CD8 staining) content ($P<0.000025$, $P<0.000001$, respectively) but not that of other cell types (E). The reduction of neutrophils and cytotoxic T cells is paralleled by a trend to reduced levels of the neutrophil marker MMP8 (A; neutrophil collagenase; $P<0.0053$, NS) and the cytotoxic T-cell activation markers perforin ($P<0.0158$, NS) and granzyme A ($P<0.00001$; F). #Significant difference between doxycycline-treated patients (gray) and nontreated control subjects (white).

Doxycycline and the Inflammatory Responses in AAA

Because of the selective effect of doxycycline treatment on MMP9 protein levels but not on mRNA expression, we next analyzed the aortic wall neutrophil content. Figure 1B and 1C show that doxycycline markedly reduced the aortic wall neutrophil content (76% reduction of the median value; $P<0.000025$). Further evaluation of other cell types in the aorta also revealed a profound reduction of the aortic wall cytotoxic (CD8+) T-cell content (96% reduction of the median value; $P<0.000001$; Figure 1C and 1D) but not on other cell types (monocytes/macrophages [CD68+]; T-helper cells [CD4+], B cells [CD20+], and plasma cells [CD138+]; Figure 1E). Figure 1B and 1D illustrate that the effects of the individual doxycycline doses are equivalent.

Tissue distribution of these cells as assessed by immunohistochemistry was not influenced by doxycycline.

The decrease in neutrophil and cytotoxic T-cell count was paralleled by a reduction of granzyme A ($P<0.000010$; Figure1F), a specific marker of cytotoxic T-cell activation. The effect on the neutrophil-specific marker neutrophil collagenase (MMP8; $P<0.0053$; Figure 1A) and the cytotoxic T-cell marker perforin ($P<0.0158$) did not reach statistical significance.

mRNA levels of markers of monocyte/macrophage activation (ie, macrophage elastase [MMP12] and cathepsin K) were not affected by doxycycline therapy. Similarly, except for a small reduction of IgG heavy-chain expression, doxycycline therapydid not influence markers of B-cell/plasma cell activity (MAD-4, BLIMP-1, and IgG linker protein; Table 2).

TABLE 3

Effect of Doxycycline Treatment on Aneurysm Wall Cytokine and Chemokine Protein Expression

	Nontreated Control Subjects, pg/mg Protein	Doxycycline-Treated Subjects, pg/mg Protein	*P**
IL-1β	4.27 (2.42–5.86)	3.50 (1.71–5.39)	0.352 (NS)
IL-2	0.28 (0.21–0.34)	0.23 (0.18–0.31)	0.389 (NS)
IL-6	462 (178–2417)	148 (63–307)	0.00115
IL-7	0.15 (0.00–0.22)	0.18 (0.11–0.29)	0.274 (NS)
IL-8	165 (92–449)	70 (34–147)	0.00246 (NS)
IL-13	1.43 (0.54–3.23)	0.57 (0.27–0.94)	0.0184 (NS)
G-CSF	6.02 (3.66–10.85)	3.48 (2.09–5.20)	0.0308 (NS)
GM-CSF	0.33 (0.00–0.69)	0.37 (0.06–0.69)	0.424 (NS)
Interferon-γ	8.16 (4.94–11.83)	5.88 (2.91–8.43)	0.109 (NS)
MCP-1	369 (211–907)	351 (190–599)	0.329 (NS)
MIP-1β	7.46 (6.10–13.48)	12.05 (6.92–18.87)	0.166 (NS)
TNF-α	0.24 (0.04–0.32)	0.17 (0.05–0.26)	0.579 (NS)

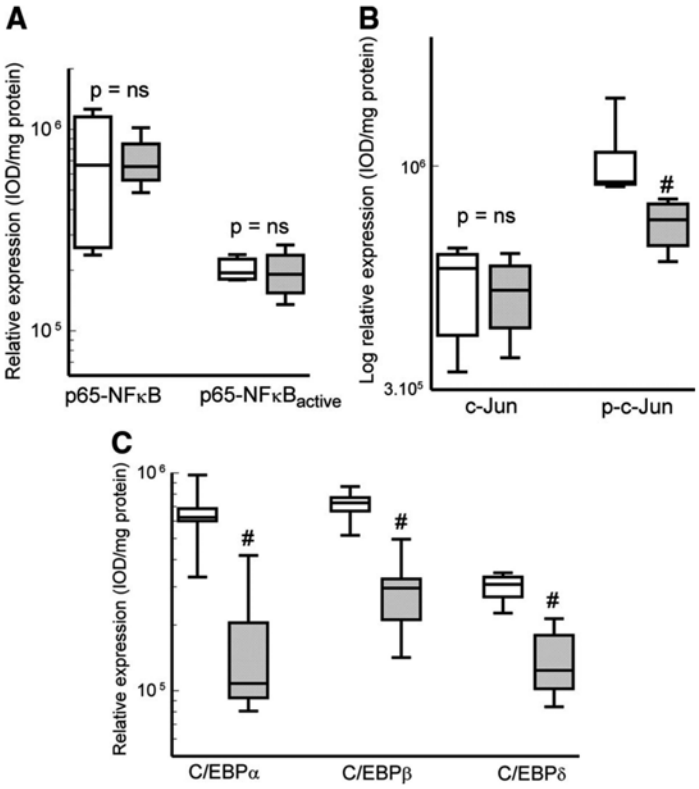
NS indicates level of significance not reached after Bonferroni-Holm's correction; GM-CSF, granulocyte macrophage CSF; MIP, macrophage inflammatory protein; and TNF, tumor necrosis factor. Values were assessed by the BioRad 17-Plex panel for multiple cytokines. IL-4, IL-5, IL-10, IL-12, and IL-17A levels were all below the detection threshold of the assay and are therefore not shown. Levels of IL-6, IL-8, and MCP-1 exceeded the upper detection threshold and were accurately quantified in separate ELISAs. Values are median (interquartile range). *Probability value is for the combined doxycycline groups versus the nontreated control group.

Effects of Doxycycline on Cytokine and Chemokine Expression

Doxycycline treatment resulted in a selective suppression of aneurysmal wall cytokine and chemokine protein levels (Table 3). IL-6 hyperexpression, a characteristic feature of AAA,[22] was reduced from median values of 462 to 148 pg/g protein (IL-6; $P<0.00115$). The reduction of IL-8 levels (median values in thecontrol group and the treated group, 165 and 70 pg/g protein, respectively) did not reach statistical significance ($P<0.00246$). The strong positive relationship between neutrotactic chemokine IL-8 and aortic wall neutrophil content in the control group ($r=0.84$, $P<0.00016$) was not found in the doxycycline-treated group ($r=-0.037$, $P<0.819$).

Consistent with the absence of an effect on monocyte/macrophage activation markers, doxycycline therapy did not influence the levels of inflammatory cytokines and chemokines that are associated predominantly with monocytes/macrophages, ie, IL-1β ($P<0.352$), tumor necrosis factor-α (0.579), MCP-1 (CCL-2; $P<0.329$), and macrophage inflammatory protein-1β (CCL4; $P<0.166$; Table 3).

FIGURE 2

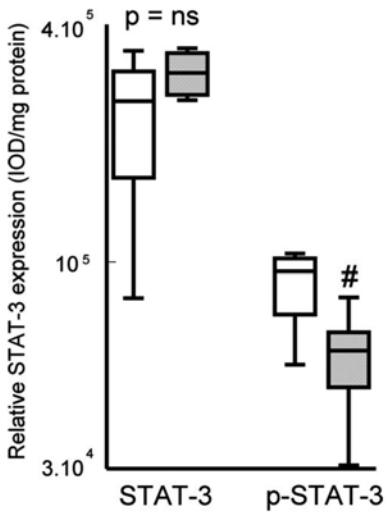


The effect of doxycycline on the NFκB, AP-1, and C/EBP inflammatory pathways. Doxycycline may reduce AP-1 activation (phospho-c-Jun; *P*<0.0158, NS) and reduces C/EBPα, β, and δ expression (all *P*<0.00117) but does not influence basal AP-1 (c-Jun) (*P*<0.905) and NFκB (*P*<1.000) levels and NFκB activation (*P*<0.730). #Significant difference between doxycyclinetreated patients (gray) and nontreated control subjects (white).

Effects of Doxycycline on Mechanistically Relevant Inflammatory Transcription Factors

Doxycycline reduced the aortic wall protein concentrations of cytokines/chemokines that are regulated by AP-1 (IL-6 and IL-8) and/or C/EBP (IL-8, G-CSF) but did not affect the level of cytokines/chemokines that are controlled predominantly by NFκB (eg, IL-1β and MCP-1). These observations suggest that doxycycline therapy specifically quenches the inflammatory transcription factors AP-1 and/or C/EBP. Evaluation of AP-1, C/EBP, and NFκB protein expression levels and their activation status (active phosphorylated c-jun for AP-1, active p65-NFκB protein confirmation for NFκB; Figure 2A and 2B) provided indications for an effect on the protein levels of all 3 C/EBP isoforms (α/β/δ; all *P*<0.00117; Figure 2C) and possibly on the activation of the AP-1 system (phosphorylated c-jun, the major effector of AP-1; *P*<0.0158, NS). Levels of inactive and active p65-NFκB, the major effector of NFκB, were not influenced by doxycycline (*P*<1.000 and *P*<0.7301, respectively; Figure 2A).

FIGURE 3



The effect of doxycycline treatment on basal STAT-3 levels and STAT-3 phosphorylation, the primary downstream effector pathway of IL-6 signaling. Doxycycline does not influence basal STAT-3 levels (P<0.3428), but a trend toward reduced STAT-3 phosphorylation (p-STAT-3; P<0.0268) suggests reduced IL-6 signaling. White indicates nontreated control subjects; Gray, doxycycline-treated subjects.

To test whether the doxycycline-mediated suppression of IL-6 levels is sufficient to impair the IL-6 signaling pathway, we evaluated the levels of STAT-3, the primary downstream transcriptional effector of the IL-6 signaling pathway. Basal STAT-3 expression levels were comparable in doxycycline-treated and nontreated individuals (P<0.3428, NS), but a trend (P<0.0268, NS) toward reduced STAT-3 phosphorylation (ie, the activated form of this transcription factor) suggests diminished IL-6 signaling on doxycycline therapy (Figure 3).

Discussion

In this clinical trial, we demonstrate that doxycycline treatment has strong, highly selective effects on vascular inflammation in the AAA. The lowest doxycycline dose tested (50 mg/d) led to a profound reduction of aneurysmal wall neutrophil and cytotoxic T-cell content, 2 cell types considered crucial for the process of aneurysm formation.[18–21]

AAA is a common pathology and a major cause of death resulting from rupture. The hallmark pathology of AAA is a localized, chronic inflammatory response accompanied by a proteolytic imbalance.[5,22,23] Increased proteolytic activity in the disease is responsible for the increased matrix turnover and progressive weakening of the aortic wall. Prominent MMP9 expression in AAA,[3,5,6] along with resistance of MMP9 knockout mice to aneurysm formation,[4] led to the concept of MMP9 inhibition as a means of reducing AAA formation and expansion.[2] Independently of their antimicrobial properties, members of the tetracycline family have been shown to inhibit MMP9 expression[7] and activity[25] and to effectively prevent tissue injury

in animal models of gingivitis, arthritis, and other inflammatory disorders.[7] On the basis of these and similar studies, it was proposed that the tetracycline analogue doxycycline may inhibit AAA progression.[2] Indeed, doxycycline has shown to forestall AAA formation and growth in various animal models of the disease.[6,8–10] Results from two preliminary human studies suggest that these experimental findings may also apply to human disease.[11,12]

Remarkably, although the rationale behind doxycycline therapy is based on its putative effects on MMP9 expression and activity,[2,6] the effects are discussed controversially in published human studies.[13,14] In the first published study, Curci et al[13] evaluated the effects of preoperative doxycycline treatment (100 mg orally twice a day for 7 days) in 8 patients with an AAA and compared the results with 7 nontreated controls. They showed that doxycycline treatment resulted in a 2.5-fold reduction of MMP9 protein levels (immunoblot) and an 82% reduction of MMP9 mRNA expression (determined by a semiquantitative method, competitive real-time polymerase chain reaction). In a later placebo-controlled study, Ding et al[14] tested the effect of 1 month of doxycycline treatment (100 mg once a day) or placebo on MMP mRNA expression (real-time polymerase chain reaction) and protein levels (both ELISA and immunocapture assays) in 51 patients scheduled for open repair. Unlike the initial study by Curci et al,[13] no effect was found on MMP mRNA or protein expression.

Our findings suggest an effect of doxycycline therapy on MMP9 protein levels ($P<0.00263$), as reported by Curci et al.[13] Yet, in line with Ding et al,[14] we did not find an effect of doxycycline on MMP9 mRNA expression levels ($P<0.206$). The reducing effect of doxycycline on MMP9 protein but not on mRNA may be explained by reduced aneurysmal wall neutrophil content. Neutrophil MMP9 is produced (transcribed and translated) during neutrophil maturation in bone marrow, and the protein is subsequently stored in the secondary granules. MMP9 mRNA expression in circulating or infiltrated neutrophils is neglectable.[15,16] The absence of an effect of doxycycline on aortic wall MMP9 mRNA expression suggests that treatment does not affect MMP9 expression in other cell types (eg, macrophages). Together, these observations may suggest that the reduction of MMP9 protein can be ascribed mainly to the disappearance of aortic wall neutrophils and that the effects of doxycycline therapy relate to an anti-inflammatory effect.

This study shows that the immune modulatory effects of doxycycline in AAA are profound and potentially therapeutically relevant and that these effects are highly selective; a brief period of 2 weeks of doxycycline treatment suffices to strongly reduce aortic wall neutrophil and cytotoxic T-cell content. These cellular effects were paralleled by reduced levels of cytotoxic T-cell–specific marker granzyme A. The effects on perforin ($P<0.0158$), another cytotoxic T-cell activation marker, and the neutrophil markers neutrophil collagenase (MMP8) and neutrophil gelatinase (MMP9) ($P<0.0053$ and $P<0.00263$, respectively) did not reach significance after Bonferroni-Holm's correction. Doxycycline did not affect the number and activation status of other prominent cell types in AAAs (ie, T-helper cells, monocytes/macrophages, B cells, or plasma cells),[22] showing that the immune modulatory effects of doxycycline are selective rather than universal.

In the present relatively small study, we did not observe a dose-response relationship for the parameters studied. All doxycycline-treated groups were well matched with respect to age, sex, and medication. We minimized well-known confounding factors that may affect bioavailability. For example, we specifically instructed patients to avoid simultaneous ingestion of doxycycline and cation-containing preparations and excluded patients with kidney dysfunction.

The absence of a dose-response relationship in our study presumably indicates that the anti-inflammatory effects in AAA are already maximal at the lowest dose of doxycycline used in this study (50 mg). Consistent with this, subantimicrobial doses of doxycycline (20 mg BID) are approved by the Food and Drug Administration for inhibition of excess MMP8 and MMP9 activity in periodontitis,[26] showing that low-dose systemic doxycycline treatment has clinical effects. For logistical reasons, the treatment period in this study was relatively short (2 weeks), yet observations from other chronic inflammatory conditions involving neutrophils such as periodontal disease, acne, and rosacae7 suggest that the effects of doxycycline persist during prolonged treatment.

A molecular explanation for the reduction of the aortic wall neutrophil content after doxycycline treatment may be the reduction of neutrophil chemoattractants such as IL-8[27] in the aneurysm wall. This notion is supported by the robust correlation between aortic wall IL-8 levels and aortic wall neutrophil content that is present in the nontreated controls and that is lost after doxycycline treatment. The possible reduction of G-CSF levels ($P<0.00308$, NS) may contribute to this effect because G-CSF reportedly stimulates survival, differentiation, and function of neutrophil precursors and mature neutrophils.[28,29] Available preclinical studies suggest that doxycycline may also dose dependently interfere with neutrophil migration.[30] Yet, the relevance of this effect has been questioned because of the results of an earlier clinical study.[31]

The selective reduction of CD8+ T cells, along with their activation markers perforin and granzyme A, after doxycycline treatment has not been reported previously. Our data demonstrate that the observed reduction of CD8+ T cells is selective because there was no effect on CD4+ T cells. To the best of our knowledge, T-cell trafficking and homing signals concur. In the absence of an effect of doxycycline treatment on CD4+ T cells, we hypothesize that the reduction presumably reflects a direct effect on CD8+ T cells. There is evidence for diverging roles of specific signaling pathways in CD8+ T-cell and CD4+ T-cell biology. For example, specific roles for the AP-1[32] and IL-6/STAT3[33] signaling pathways have been reported in the context of cytotoxic T-cell proliferation (AP-1) and survival (IL-6/STAT3). In the absence of known direct toxic effect of doxycycline on cytotoxic T cells,[17] we speculate that the selective reduction of cytotoxic T cells on doxycycline treatment may be mechanistically linked to the reduced AP-1 and/or STAT-3 activation after doxycycline therapy.

Direct inhibition of the AP-1 pathway has been shown to prevent and even reverse aneurysm formation in animal models of aneurysmal disease.[34] This published report showed not only

that AP-1 is critically involved in the inflammatory cascade and the proteolytic imbalance in AAA but also that reduced AP-1 signaling resulted in increased matrix deposition.

We found that doxycycline therapy reduced aortic wall G-CSF levels. G-CSF expression is under primary control of C/EBP transcription factors. All 3 isoforms (α, β, δ) are elevated in AAA,[22] and increased aortic C/EBP expression is positively associated with aortic wall inflammation in atherosclerotic occlusive disease.[35] Anti-inflammatory strategies that reduce C/EBP expression have been described to also reduce atherosclerotic disease.[36] Yet, little is known about the pathophysiological role of C/EBP in the context of AAA, and further mechanistic studies are necessary to investigate the contribution of C/EBP to AAA growth.

Conclusions

We report that a period of 2 weeks of doxycycline treatment in patients with advanced abdominal aneurysms results in a profound and selective reduction of aortic wall neutrophil and cytotoxic T-cell content. This cellular effect is paralleled by a selective suppression of inflammation on the molecular level, ie, cytokines (IL-6, IL-8) and transcription factors (AP-1, C/EBP, STAT3), which are relevant for neutrophil and cytotoxic T-cell inflammation, as well as by a specific reduction of neutrophilderived proteases. Our findings show that doxycycline therapy may be a useful strategy to selectively quench specific cellular and molecular aspects of vascular inflammation in AAA. The ability of doxycycline to reduce aneurysmal growth awaits confirmation in a sufficiently powered clinical trial. These data provide a promising avenue for further research evaluating the efficacy of doxycycline in other chronic vascular and nonvascular inflammatory conditions involving neutrophils and/or cytotoxic T cells such as Kawasaki disease[37] and chronic obstructive pulmonary disease.[38]

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Silent road, 2008

Distinct defects in collagen microarchitecture underlie vessel-wall failure in advanced abdominal aneurysms and aneurysms in Marfan syndrome

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Abstract

An aneurysm of the aorta is a common pathology characterized by segmental weakening of the artery. Although it is generally accepted that the vessel-wall weakening is caused by an impaired collagen metabolism, a clear association has been demonstrated only for rare syndromes such as the vascular type Ehlers–Danlos syndrome. Here we show that vessel-wall failure in growing aneurysms of patients who have aortic abdominal aneurysm (AAA) or Marfan syndrome is not related to a collagen defect at the molecular level. On the contrary our findings indicate similar (Marfan) or even higher collagen concentrations (AAA) and increased collagen cross-linking in the aneurysms. Using 3D confocal imaging we show that the two conditions are associated with profound defects in collagen microarchitecture. Reconstructions of normal vessel wall show that adventitial collagen fibers are organized in a loose braiding of collagen ribbons. These ribbons encage the vessel, allowing the vessel to dilate easily but preventing overstretching. AAA and aneurysms in Marfan syndrome show dramatically altered collagen architectures with loss of the collagen knitting. Evaluations of the functional characteristics by atomic force microscopy showed that the wall has lost its ability to stretch easily and revealed a second defect: although vascular collagen in normal aortic wall behaves as a coherent network, in AAA and Marfan tissues it does not. As result, mechanical forces loaded on individual fibers are not distributed over the tissue. These studies demonstrate that the mechanical properties of tissue are strongly influenced by collagen microarchitecture and that perturbations in the collagen networks may lead to mechanical failure.

Introduction

Aortic aneurysms are localized dilatations of the aortic wall that are caused by segmental weakening of the vessel wall. Although aneurysms generally are without clinical symptoms, larger aneurysms may rupture, and bleeding from a ruptured aneurysm is responsible for more than 15,000 annual deaths in the United States alone.[1] Aneurysm formation relates to a primary or secondary (acquired) defect in the matrix structures supporting the vessel wall resulting in attenuation and ultimate failure of the vessel wall.[2] Although extensive loss of medial elastin traditionally is considered the hallmark of aneurysm formation, it now is acknowledged that aneurysmal growth and ultimate rupture relate to impaired collagen homeostasis.[2] Remarkably, although numerous studies have looked for putative quantitative changes in aortic collagen, results reported to date are controversial.[3–5] With the exception of rare mutations in the collagen III gene such as the vascular type of Ehlers–Danlos syndrome, no clear association between impaired collagen homeostasis and aneurysm growth and/or rupture has been identified. In search of the collagen defect(s) underlying aneurysm formation, we applied an integrated approach of biochemical analyses, multiple imaging modalities, and functional analysis by atomic force microscopy (AFM) to identify the putative collagen defect in aortic abdominal aneurysm (AAA) and in Marfan syndrome, by far the two most common forms of aortic aneurysms. Results of this evaluation show that advanced stages of aneurysmal disease are characterized by distinct defects in the adventitial collagen skeleton armoring the vessel wall rather than by purely biochemical defects.

Materials and methods

All human arterial wall samples were provided by the Vascular Tissue Bank, Department of Vascular Surgery, Leiden, The Netherlands. Sample collection and handling was performed in accordance with the guidelines of Medical Ethical Committee of the Leiden University Medical Center, Leiden, The Netherlands, and the code of conduct of the Dutch federation of Biomedical Scientific Societies.

Anterior lateral aneurysm wall samples were obtained from patients with an AAA >55 mm undergoing elective open repair (AAA group: n = 17,mean age 72.4 ± 6.2 years). Thoracic aortic aneurysms (ascending aorta, diameter >50 mm) of Marfan patients were obtained during elective repair (Bentall procedures) (Marfan group: n = 11, age 26.9 ± 8.2 years). All Marfan patients met the international criteria for Marfan syndrome.[6]

Control (normal) abdominal aortic wall was obtained during kidney explantation for organ donation. All these control samples were obtained from the level of the renal artery, i.e., from a location comparable to that of the samples from AAA patients (n = 11, age 55.6 ± 10.2 years). The primary cause of the fatal brain injury in this control group was a major head trauma or subarachnoidal bleeding.

Control thoracic aorta (post mortems) from patients dying from noncardiac causes was used as a histological reference for the Marfan tissue. Following excision, half of the sample was fixed in formalin for 24 h, decalcified in Kristensen's solution, and subsequently embedded in paraffin for immunohistochemical analysis. The remaining half was immediately flash-frozen in liquid nitrogen for mRNA analysis and for the preparation of cryosections.

Collagen and Cross-Link Analysis

Ten 10-µm slices of paraffin-embedded tissue were deparafinized in xylene, and the samples were hydrolyzed (110 °C, 20–24 h) in 1 mL 6 M HCl in 5-mL Teflonsealed glass tubes. The samples were dried and redissolved in 1 mL of water containing 10 µmol/L of pyridoxine [internal standard for the cross-links hydroxylysylpyridinoline (HP) and lysyl-pyridinoline] and 2.4 mmol/L of homoarginine (internal standard for amino acids) (Sigma). Samples were diluted 5-fold with 0.5% (vol/vol) heptafluorobutyric acid (Fluka) in 10% (vol/vol) acetonitrile for cross-link analysis; aliquots of the 5-fold diluted sample were diluted 50-fold with 0.1 M sodium borate buffer (pH 8.0) for amino acid analysis. Derivatization of the amino acids with 9-fluorenylmethyl chloroformate and reversed-phase HPLC of amino acids and crosslinks was performed on aMicropakODS-80TMcolumn (150mm×4.6 mm) (Varian) as described previously.[7,8] The quantities of the cross-link HP were expressed as the number of residues per collagenmolecule, assuming 300 hydroxyproline (Hyp) residues per triple helix. This procedure is well established, because Hyp is a collagen-specific amino acid and because the prolyl hydroxylation level in collagen is stable.

RNA Extraction and mRNA Analysis

Total RNA extraction was performed using RNAzol (Campro Scientifics) and glass beads

according to the manufacturer's instructions. Copy-DNA was prepared using kit #A3500 (Promega), and quantitative realtime PCR analysis was performed for collagen type I and III and lysyl oxidase on the ABI-7700 system (Applied Biosystems) using established primer/probe sets (Assays on Demand; Applied Biosystems) and MasterMix (Eurogentec). Analyses were performed according to the manufacturers' instructions and as previously reported.[9] GAPDH expression was used as a reference and for normalization.

Histology

Histochemistry and immunohistochemistry was performed on 4-μm deparaffinized, ethanol-dehydrated tissue sections. Collagen staining was performed by the Sirius Red-picrine method.[10] Immunohistochemical staining for collagen type I and III and fibrillin was performed using specific antibodies for collagen type I (C7510-17K; US Biological), collagen type III (C7510-39G; US Biological), or fibrillin (MAB1919, Chemicon). Pepsin-trypsin retrieval was required for optimal collagen I and III staining, and a 10-mM (pH 6.0) citrate retrieval was required for the fibrillin staining.

AB-conjugated biotinylatedanti-goat or rabbit anti-IgG was used as secondary antibody. Sections were stained with NovaRed(Vector Laboratories) and counterstained with Mayer's hematoxylin. Controls were performed by omitting the primary antibody.

We used confocal microscopy (LSM615; Zeiss) for the visualization of the collagen network structures. Tissue slices (20 μm) were stained with Sirius Red, and Sirius Red fluorescence was used for the reconstruction. Fibrillin deposition was evaluated by immunohistochemical staining (see above) using the Alexa 647 mouse anti-goat antibody for visualization. Serial confocal sections of x-y images (stack size 206 × 206 μm) of representative sections of the medial and adventitial layer were made along the z axis with a distance of 0.5 μm. The excitation and emission wavelengths were 543 and 633 nm, respectively, for the Sirius Red staining and were 633 and 647 nm, respectively, for immunohistochemical (Alexa 647) staining. The pinhole was set at 108 μm. Three-dimensional reconstructions of confocal stacks (20 serial images at 0,5-μm intervals) were performed using the stacks- Z-function on the Zeiss LSM Image Examiner version 3.2.0.115.

Atomic Force Microscopy

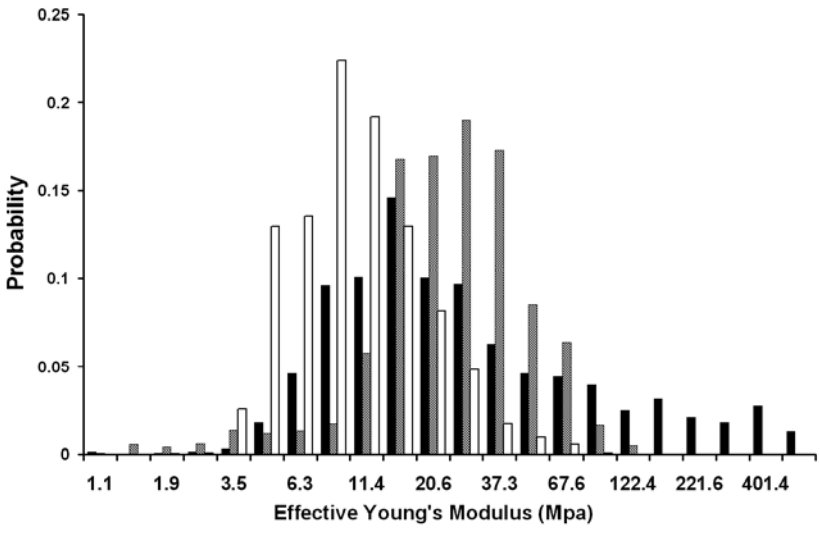
Atomic force microscopy (AFM) measurements were performed on 8-μm-thick tissue slices. To that end, tissue was cryosliced to 8-μm thickness, perpendicular to the flow of the blood, and stored at -80 °C. Measurements were done using a Molecular Imaging PicoScan atomic force microscope controlled with a custom scripting program written in Labview (National Instruments) and Visual Basic 6 (Microsoft). Data were recorded with a National Instruments card at 100 kS/s and then processed into higher-resolution force–volume images than can be taken with the control software of the AFM. All force–distance curves were performed at a rate of 1 Hz.

Tissue was probed at two different resolutions: A sharp tip (20-nm tip radius) was used to probe the tissue on the fibril level, anda 10-μm ball tip was used to probe at the tissue level.[11,12] The sharp tips were Nanoprobe tips model NP (Veeco). Cantilevers were calibrated using the thermal method.[13]

Balls tips were specially modified tips from Novascan. These tips are Park cantilevers (Veeco) to which a 10-μm glass ball is attached. Because the effective stiffness of the tissue is greater when probed at a larger scale, we choose cantilevers with a force constant of 0.32 N/m. Tips were coated by a layer of polyethylene glycol (molecular weight 3,400 kDa) to prevent fouling.

All AFM measurements were performed in a liquid cell (Molecular Imaging) in PBS. Preliminary evaluation showed that the AFM measurements on the aorta sections were independent of the spring constant of the cantilevers. Consistency of the data was checked by measuring the variations in stiffness in a number of healthy individuals. As shown in Figure 1, the variation among the individuals tested is consistent with the conclusion that the differences found among different tissues are disease specific rather than representing variation among individuals.

FIGURE 1



Force distribution of three distinct healthy aortas (black, gray, and white bars) measured by the sharp tip.

AFM Function and Nano indentation

Force–extension curves were performed by laying a section of tissue on a glass slide and immersing it in the PBS buffer in the liquid cell. Tissue then was loaded in the AFM microscope, and a force–volume dataset was produced by pushing the cantilever into the tissue at each point on a grid. This technique allowed the collection of a 2D grid of indentation data that then was transformed as explained below into effective Young's modulus as well as a constant-force topography.

The Young's modulus was determined using the methods of A. Hassan et al.[14] This technique is more robust to the errors that are inherently present in fitting the Hertz model[15] of an indenter to data collected from AFM force–distance curves. A detailed discussion of the methods that can be used to find the Young's modulus from AFM force–extension curves can be found in Stolz et al.[16] or in A-Hassan et al.[14] This technique involves determining the work performed by the cantilever on the tissue sample and then comparing this work with a known standard. By integrating the total area under the force–distance curve, local variations are minimized, and the curve fitting becomes less difficult. Most importantly, the exact point of contact between surface and AFM cantilever is no longer needed, because the area under the curve at the estimated contact point is small compared with the total area under the curve. Its ease of application and, more importantly, its reproducibility, make this technique the more desirable method of determining the effective Young's modulus of the tissue.

In this case, Sneddon's equations[17] were used to estimate the curve that would be expected with a conical (sharp tip) or spherical (ball tip) Hertz model indentor. Although this estimation negates some of the advantages of this method, namely the ability to divide the shape, force constant, and sensitivity of the tip from the equation, it still produces a standard curve to which all of the curves analyzed by this method have been compared. Thus, although the absolute value for the Young's modulus has a large error, the error is systematic, and comparisons between the various datasets are still completely valid.

Validity and Reproducibility of the AFM Measurements

Two different force constants were used between the ball tips and the sharp tips. To determine what effects the differing force constants would have, we performed an experiment with the sharp tip on aortic abdominal aneurysm (AAA) tissue. Various cantilevers were available on the nanoprobe chip; we used cantilevers with the 0.06-N/m and the 0.32-N/m tips, force constants that were chosen specifically to match the stiffness of the tissue and therefore to maximize the force resolution.

The effect from the force constant of the cantilever over this range is negligible. The observed differences pale in comparison with the differences between the Marfan and AAA tissue. Therefore we can safely assume that the differences seen in the aneurysmal tissue between the ball tip and the sharp tip result solely from the differences in tip shape.

Action of Ball Tip vs. Sharp Tip

The different-sized tips allow very different information about the collagen networks to be collected, and the data provide a sense of the coherence of the matrix network. The two tips allow an understanding of the collagen networks as well as interacting directly with individual proteins. The sharper tip is smaller than the radius of a collagenfibril. It is able to interact directly with these structures in the extracellular matrix as well as slip between the fibers. The ball tip was chosen to interact with the tissue on a micrometer scale. This tip is able to push tens of fibers and directly measures the network indentation properties of the tissue. The size is a compromise between seeing the large network behavior and yet remaining small enough to handle with an AFM tip.

Nano indentation Stiffness Maps

The force-vs.-distance curves were processed into a combination topography and stiffness image by combining all the force–extension curves from the AFM measurements into a grid and then taking the extension at which the force reached a certain level as the intensity at that point. This method is sensitive to both the initial height of the tissue and to the stiffness of the tissue under the cantilever. Regions where the tissue is stiffer will indent less before the required force is reached and will therefore appear higher than regions that were equally high before indentation but that are softer. We find that these images provide information complementary to the collagen confocal images.

Statistics

Differences between the groups were evaluated by an unpaired t test (for the normally distributed data) or by the Wilcoxon–Mann–Whitney test (for non–normally distributed continuous data). The level of significance was set at $P < 0.05$. All analyses were performed using SPSS16.0 (SPSS Inc.)

Results

Vascular load-bearing collagen is composed of highly stable type I and III fibrillar collagens that are stabilized further by intramolecular cross-linking.[18] Biochemical as well as morphometric evaluation showed similar collagen concentrations in aneurysm wall from patients with Marfan syndrome and normal, non-aneurysmal control aorta, whereas elevated collagen concentrations were found in AAA (Figure 2A). The ration of type I/III collagen mRNA expression was similar in control aorta, Marfan syndrome, and AAA (Figure 3). Expression of lysyl oxidase was higher in the aneurysm wall from patients with Marfan syndrome ($P < 0.05$; Figure 3). Evaluation of intermolecular collagen cross-linking through quantification of nonreducible lysyl oxidase-initiated collagen cross-links (hydroxylysyl pyridinoline/lysyl pyridinoline cross-links)[19] showed increased intramolecular collagen cross-linking in the aneurysmal wall in both AAA and Marfan tissue (Figure 2). These biochemical findings for AAA are in line with reports in the available literature, and none of the findings indicates a quantitative or qualitative defect in vascular collagen at the molecular level in AAA or in aneurysms of patients with Marfan syndrome.

In the absence of a clear defect at the biochemical level, we sought for possible structural defects in collagen organization. Histological evaluation (Picrosirius Red collagen staining) (Figure 2B) and immunohistological staining for collagen type I and III (Figure 4) show distinct differences in collagen organization in the medial and adventitial layers of the (grossly) normal aortic wall. A similar pattern but with minor fibrotic changes is seen in the aneurysm wall of patients with Marfan syndrome (Figure 2B and Figure 4). Collagen deposition in AAA, on the other hand, is hallmarked by complete loss of vessel-wall architecture and deposition of disorganized and condensed collagen (Figure 2B and Figure 4), a finding that is consistent.

Because the regular 2D images may mask structural defects in the third dimension,[20] we also created 3D reconstructions of the medial and adventitial collagen microarchitecture using the Z-stack function on the confocal microscope. These reconstructions show a clearly distinct collagen organization in the medial and adventitial layers of the normal aortic wall (Figure 5). Collagen deposition in the medial layer is best characterized by small, interdispersed collagen fibrils that run mainly perpendicular to the circumferential elastic sheets (Figure 5A). Adventitial collagen, on the other hand, is arranged in a loose knitting of highly organized ribbon-like collagen bands that brace the medial and intimal layers of the vessel wall (Figure 5B). These different architectures appear optimal for achieving the different functionalities for the aortic medial and adventitial layers (elastic recoil and resilience, respectively).[21,22]

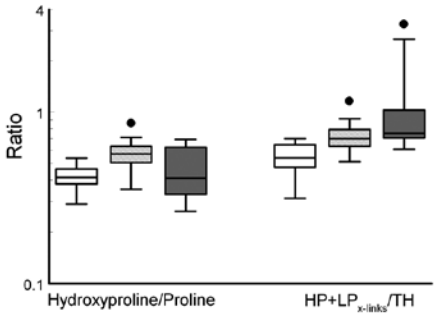
Evaluation of collagen architecture in aneurysms of patients with Marfan syndrome showed minor changes in the medial layers of the aortic wall (Figure 5C) but a dramatically disturbed collagen architecture in the adventitial layer, with complete absence of the normal collagen fibril organization and deposition of thin parallel collagen fibrils (Figure 5D).

Reconstructions for the AAA (Figure 5E) show complete loss of the normal architecture, loss of the distinction between medial and adventitial collagen organization, and deposition of aggregated, parallel collagen sheets that appear rigid.

The disordered properties of the collagen microarchitecture in AAA and in the adventitial layer of aneurysms from patients with Marfan syndrome suggest that defects in adventitial collagen organization underlie the weakening of the aortic wall. To test this hypothesis, we performed functional analysis by AFM on normal and aneurysmal adventitial aortic sections. AFM is an established means to test the mechanical properties of individual proteins, cells, and tissue.[23,24] AFM experiments are performed by indenting the tissue at multiple points with a needle and testing the mechanical response.[25] The tissue response to the indentation then is used to calculate the elasticity (Young's modulus) for the given point. Multiple individual elasticity measurements then are integrated to create a visual representation (stiffness map) of the probed area. The high force resolution of the AFM (in pico-newtons) and its high lateral resolution (i.e., several elasticity measurements per square micrometer) makeAFM ideal for precisely mapping the mechanical properties of tissue at the microscale level, thereby allowing comparison of the elasticity map with the confocal images (Figure 5).

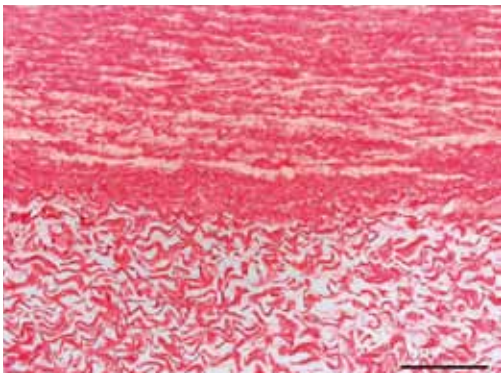
FIGURE 2

A

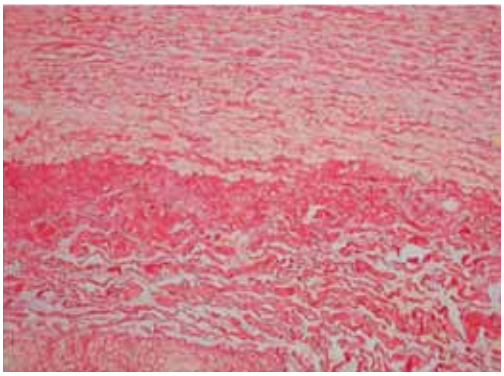


(A) Aortic wall collagen levels were assessed by the hydroxyproline/ proline ratio, and the extent of intramolecular collagen cross-links was assessed by the number of hydroxylysyl pyridinoline and lysyl pyridinoline cross-links per triple helix. Open box, control; light gray box, Marfan syndrome; dark gray box, AAA; •, $P < 0.00001$.

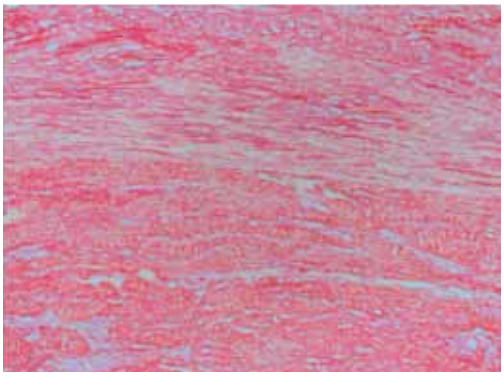
B



Normal



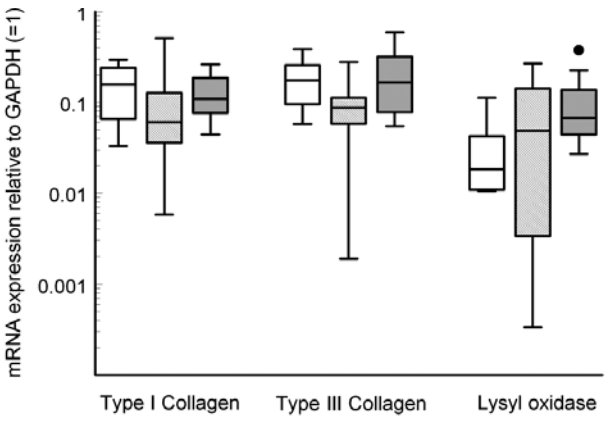
Marfan



AAA

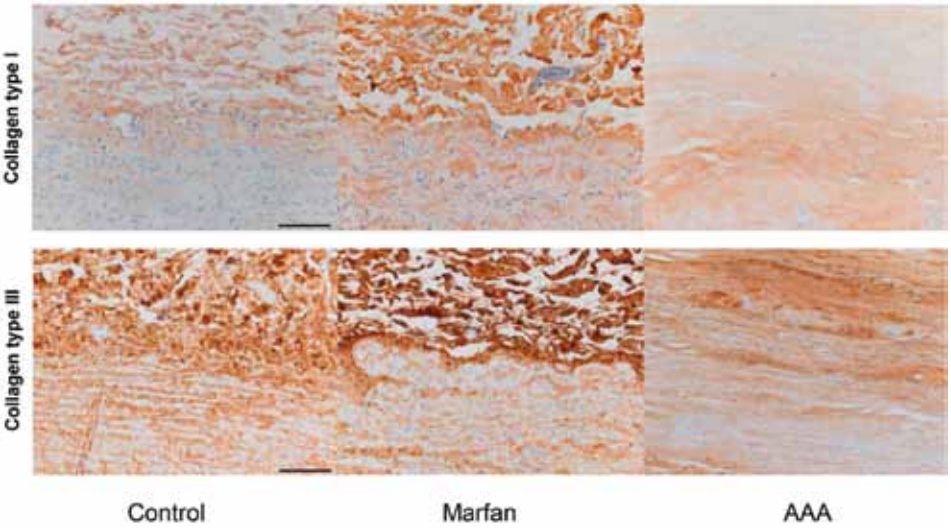
(B) Collagen deposition (Sirius Red staining) in normal aortic wall and aneurysmal wall in Marfan syndrome and AAA. The images are oriented with the media layer in the upper half and the adventitial layer in the lower half.

FIGURE 3



mRNA expression relative to GAPDH (GAPDH = 1) of type I and III collagen, and lysyl oxidase (a key enzyme required for collagen cross-linking). Open box, Control; light gray box, Marfan syndrome; dark gray box, AAA; •, P < 0.00001.

FIGURE 4



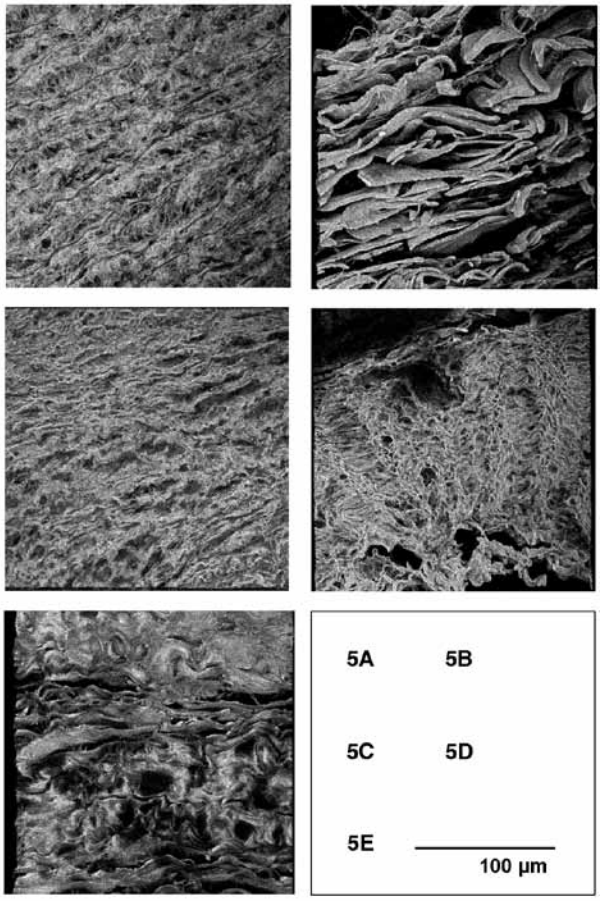
Collagen type I and III distribution in medial-adventitial border zone of control (abdominal) aorta, ascending aortic aneurysm in Marfan syndrome, and AAA. (Scale bar, 100 μM.)

Moreover, the individual elasticity measurements can be compiled into a histogram that can be used to express the distribution of the elasticity of the arterial wall at a larger scale.

Tissue (adventitial layer) was probed at two different levels by using different AFM cantilevers: a sharp tip (20-nm end radius) and a blunter ball tip (10-μm radius). These two tips allow

a comparison of the different scales in the tissue. The size of the sharp tip is chosen to interact only with individual molecules in the tissue (individual fiber level), whereas the larger ball tip is designed to probe at the tissue level (fibril behavior). The elasticity modulus (Young's modulus) was calculated for each indentation, because then the size and shape of the indenter can be removed, allowing direct comparison of the two measurements. The combined measurements provide a complete picture of the mechanical properties of the tissue and allow the AFM measurements to be compared with previous conventional studies of the mechanical properties of AAA and normal aorta segments.[26,27] Note, however, that all measurements are performed on a microscale level, not fully loading the fibers.

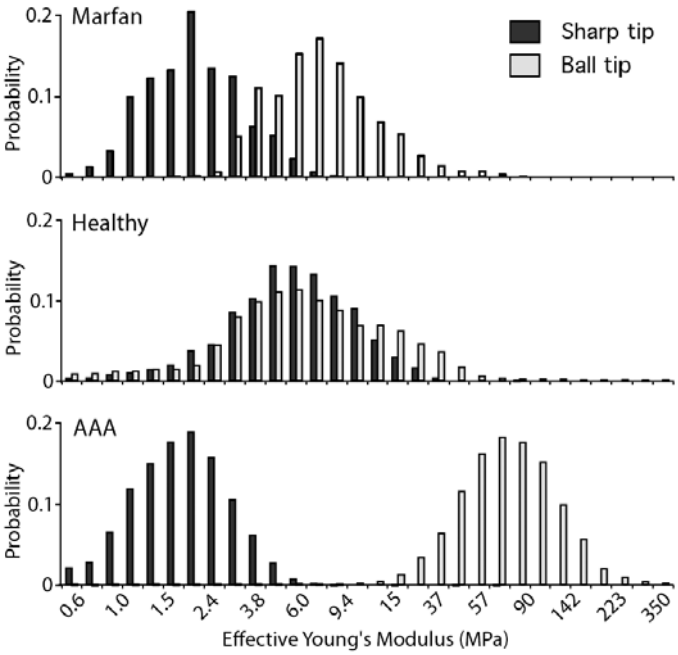
FIGURE 5



(A and B) Three-dimensional reconstructions of collagen networks in the normal media and adventitial layer. (C and D) Aneurysms in patients with Marfan syndrome. (E) Aortic abdominal aneurysm.

Stiffness histograms combining the individual data points show that the tissue response of the control adventitia is independent of the size of the tip that is used. This finding indicates that the normal adventitial tissue behaves as a highly coherent network. The stresses encountered by the tissue are dispersed equally over the whole network, and the scale of the challenger does not matter. The fact that the sharp indenter is not able to penetrate the network to a greater extent than the ball tip shows that the individual collagen fibers are densely interconnected, because the fibers do not slide out of the way and are all pulled when a single fiber is moved.

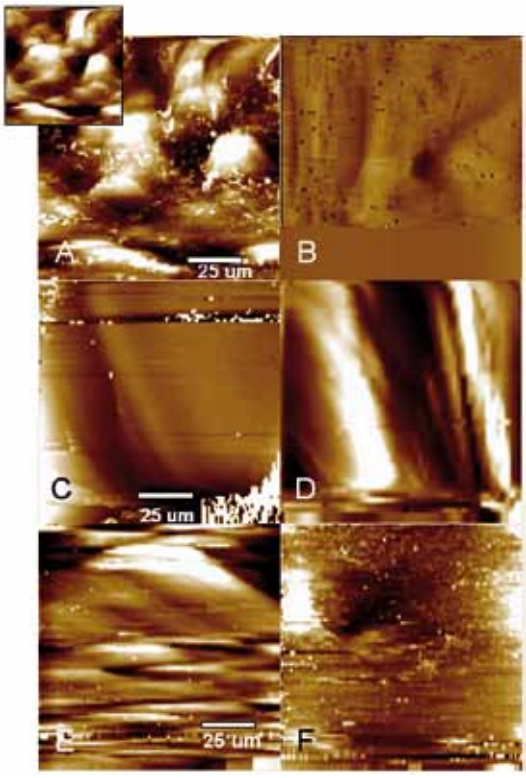
FIGURE 6



Histograms showing the distribution of the effective Young's modulus for the different conditions. Black bars show curves taken with the sharp tip; white bars show curves taken with the ball tip. Values are plotted on a log scale to improve the comparison of the modulus.

The major difference between the aneurysmal tissues and normal tissue is immediately apparent from the histogram in Figure 6 and the AFM force–volume spectroscopy (Figure 7). Unlike normal tissue, the stiffness of AAA tissue is clearly dependent on the size of the AFM tip: The ball tip, which interacts with the larger structures, senses a very stiff tissue. This observation is in line with our confocal images and conventional biomechanical studies that show that AAA tissue has become stiffer.[28] The sharp tip, on the other hand, meets hardly any resistance, suggesting that the sharp tip pushes the fibers of the extracellular matrix aside. This observation indicates that the interconnections that normally allow the tissue to behave as a coherent network are missing in AAA.

FIGURE 7

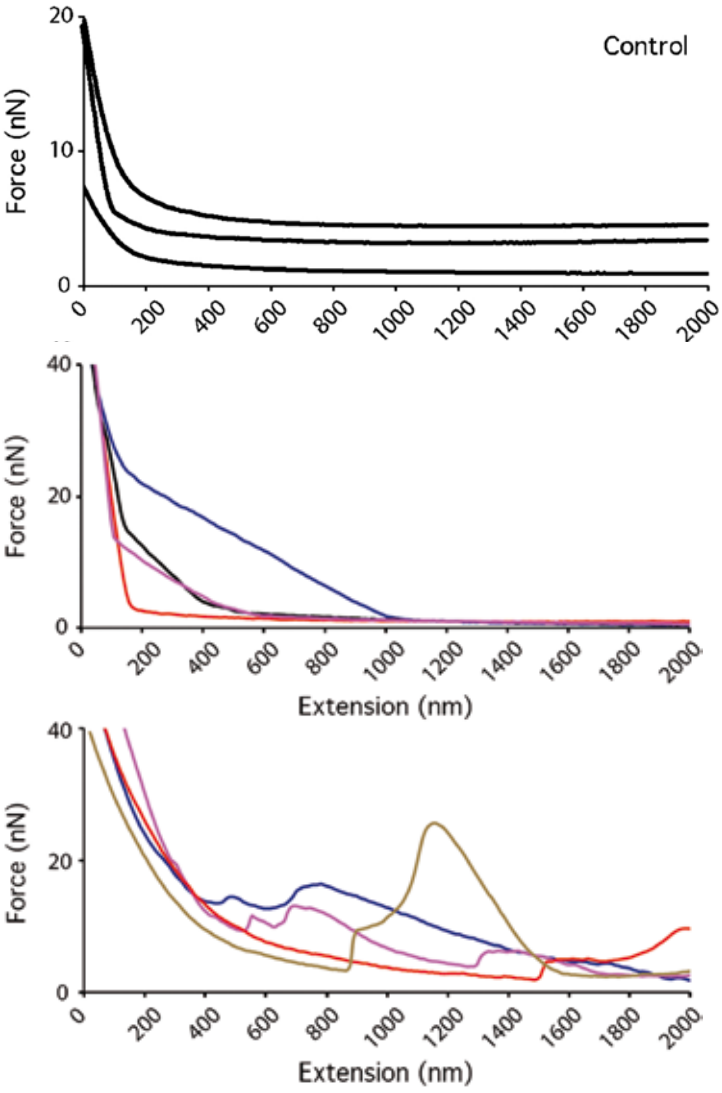


Topography maps for the various tissues as determined from AFM force–volume spectroscopy at 11 nN of constant force. Marfan tissue probed with the pyramidal tip (A) and sharp tip (B). The inset in A shows the Marfan tissue at 40 nN; it is apparent that the force collapses something in the tissue at various points resulting in a much smoother-looking surface. Healthy tissue probed with pyramidal tip (C) and ball tip (D). AAA tissue probed with pyramidal tip (E) and ball tip (F).

Probing of Marfan aneurysmal tissue shows a tissue behavior that is clearly distinct from both control aorta and AAA tissue. The larger ball tip senses stiffness similar to that of normal tissue, suggesting that under the conditions of the AFM experiment (a resting, nonstretched state) the tissue elasticity at a larger scale equals that of normal aortic wall. The sharp tip, on the other hand, finds little resistance and plunges through the tissue just as it did with AAA tissue. This observation indicates that the individual fibers are pushed aside when probed with the 25-nm tip and thus that the loaded fibrils are unable to transfer the stress and strain to the neighboring fibrils (i.e., absence of network behavior).

Another remarkable finding distinct to the Marfan tissue is that sections of the tissue collapse as the force is applied (Figure 8). These sections appear to be small voids in the collagen network that also are observed in the confocal images (Figure 5D). Such voids could provide sites susceptible to dissection or rupture.[29]

FIGURE 8



Individual force extension curves. "Extension" refers to the length of the piezoelectric tube. Motion toward zero results in indentation of the tissue by the cantilever tip. (Top) Curves sampled from control tissue. (Middle) Curves sampled from AAA tissue showing the nonlinear interactions common on the AAA tissue with the ball tip. It is proposed that the nonlinearity is a result of one collagen fiber being pushed into a second fiber, resulting in markedly stronger forces with nonlinear transitions at the connection. This behavior is consistent with the notion that there are fewer interconnections between the various collagen fibers resulting in no warning for the second fiber and its sudden recruitment. (Bottom) Curves sampled from Marfan tissue showing the collapses that are common with the Marfan tissue. These collapses could be the result of the weakened collagen fibers slipping to the side of the tip or could indicate structural weaknesses in the tissue. The confocal images in Fig. 5 A and B show the presence of small voids.

Discussion

This study shows that advanced aneurysms in AAA and Marfan syndrome are associated more with distinct defects in the collagen microarchitecture than with a collagen defect at the biochemical level. These architectural defects result in loss of the normal stress–strain curve and in impaired collagen network behavior, both of which can contribute to the aortic wall failure.

Visualization of adventitial collagen structures in the control aorta shows a collagen architecture that is best described as a loosely knitted network of interwoven collagen ribbons encasing the medial layer. A similar architecture has been described previously for the adventitial layer of the urine bladder, and it was shown that the collagen ribbons align during bladder filling, thereby allowing the bladder to distend easily but preventing overstretching.[30] The aortic adventitial layer may serve a similar purpose: Its flexibility allows the arterial wall to dilate easily but resists overstretching once fully loaded. Such a construction is similar to the textile and metal plies in a steel-belted radial tire that allow flexibility but prevent failure in extreme conditions.

The different architectures of the medial and adventitial layers may well explain the J-shaped stress–strain curve (i.e., the nonlinearity of the curve) of the normal vessel wall and suggest that the two layers have different functionalities, the elastic medial layer being responsible for the flat, horizontal part of the curve and the adventitial knitting resulting in the steep arm of stress–strain curve.[31]

Visualization of the collagen braiding in Marfan aneurysms and AAA in vessel-wall samples that were obtained at the time of operation (i.e., from advanced stages of the disease) shows that the collagen fibrils run almost in parallel, thereby limiting their ability to stretch, and therefore stiffen the vessel. This observation is well in line with biomechanical studies that show that both AAA and Marfan aneurysms are stiffer than the normal vessel wall.[18,32]

The increased stiffness of AAA tissue also is immediately apparent from the AFM experiments performed with a ball tip that show a sharp increase in the effective Young's modulus for AAA tissue, indicating that the tissue resisted even the minimal indentations (2–3 μm) by the AFM tip. The similar Young's moduli for ball-tip experiments in normal aorta and aortas from patients with Marfan syndrome seemingly conflict with biomechanical studies that indicate that Marfan tissue is stiffer than normal tissue.[32] This apparent contradiction presumably reflects a limitation when AFM measurements are performed under resting, nonstretched conditions. The confocal images clearly show that under such conditions collagen fibrils in Marfan tissue adopt a wave-like pattern that allows them to stretch when probed with the AFM tip.

Findings from this study also point to defects in collagen network behavior in the aneurysmal tissues studied. Network behavior has long been recognized as a key to mechanical stability in the field of structural design, but, remarkably, in the biomedical context network behavior

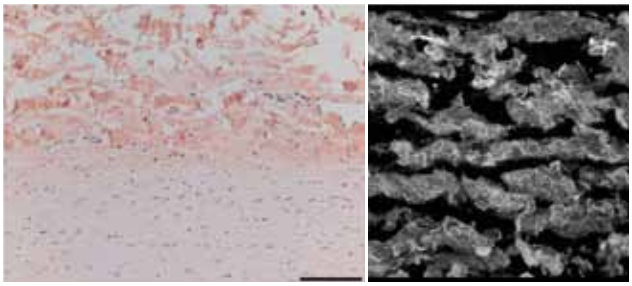
has only been reported for bone tissue.[24] Our results indicate that aneurysms in both AAA and Marfan tissue are associated with defects at all three scale lengths (i.e., at the intrafibril, intrafiber, and suprafiber levels). Impaired network behavior may interfere with the dissipation of the mechanical forces over the arterial wall, thereby contributing further to the mechanical failure of the aortic wall.

In conclusion, the findings in this study provide a structural explanation of how biological tissue (i.e., normal arterial wall) can acquire its typical nonlinear stress–strain curve and also can settle the longstanding controversy regarding the existence of a collagen deficiency in AAA. We show that defects in collagen architecture and network behavior, rather than a defect at the molecular level, explain the debilitation of the aortic wall in AAA and aneurysms in Marfan syndrome. The observed changes in AAA do not necessarily reflect the primary cause of AAA formation. More likely, the changes in AAA reflect inappropriate collagen deposition (fibrosis) in an environment that is characterized by sustained inflammation and activation of multiple proteolytic pathways. The findings in Marfan syndrome, on the other hand, may reflect a primary defect: Unlike AAA, aneurysms in patients with Marfan syndrome do show signs of increased inflammation and proteolytic activities.[33] As such, the observed defects in the collagen microarchitecture may reflect a primary defect that could relate to impaired TGF-β signaling. However, the strong link between a defect in the fibrillin gene and development of Marfan syndrome also may reflect a role of fibrillin in the organization of the collagen bands in the adventitial layer. This notion is supported by the fact that fibrillin is localized predominantly in the adventitia of the normal aortic wall (Figure 9A) and by the pattern of fibrillin deposition that is similar to that of the adventitial collagen network (Figure 9B). Our findings confirm the longstanding assumption that Marfan syndrome is a collagen disorder[34] and show that Marfan syndrome is associated with a defect in collagen network organization. Such a defect may well explain most of the other phenotypical features of Marfan syndrome which appear related to collagen dysfunction (e.g., skeletal deformities, hernias, dural ectasia, and ectopia lentis).

We speculate that defects similar to those in AAA explain the longstanding apparent contradiction between increased collagen content but reduced mechanical strength during wound healing and scar formation.[35] Microarchitectural defects in collagen network formation may well contribute to scar formation in post fetal wound healing that currently is attributed to a reduced elastin transcription after birth.[36]

FIGURE 9

A B



Fibrillin localization in the normal aortic wall. (A) Fibrillin (reddish-brown) is present primarily in the adventitial layer of the aortic wall. (B) 3D reconstruction by confocal microscopy showing that fibrillin colocalizes with adventitial collagen.

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Judgment, 2007

Summary and future perspectives

The development (initiation) of abdominal aortic aneurysms (AAAs) is caused by as yet a cascade of only partially defined pathological changes in the aortic wall. The process of AAA *progression* is driven by chronic inflammation of the aortic wall and proteinase-mediated degradation of the extracellular matrix.[1,2] It has been well documented that the strength of the aortic wall depends on the collagen network in the vessel wall.[3] Therefore we hypothesized that strategies preserving the integrity of the collagen structures will stabilize the aneurysm wall, and so reduce aneurysm progression.

Structural collagen within the arterial wall is predominantly type I and III fibrillar collagen that is highly resistant to proteolytic degradation.[3] Proteolysis of these types of collagen requires the action of specific collagenases that are able to cleave the native triple helical region of fibrillar collagens. In **Chapter 2,** we demonstrated excess turnover of fibrillar collagen in AAA. We identified members of the cysteine protease family, cathepsin K, L, and S, along with neutrophil collagenase (MMP-8), as the culprit collagenases in AAA disease. Moreover, we found a secondary deficiency of cystatin C (the primary extracellular inhibitor of cysteine proteases). We present evidence for a mechanism in which the inhibitor of one class of proteases is degraded by proteases from a different class; i.e cystatin C can be degraded by the MMPs 8 and 9 as well as by the serine protease neutrophil elastase, indicating crosstalk between different classes of proteases.

The tetracycline antibiotic doxycycline has been shown to inhibit both MMP production and activity. Doxycycline has an excellent safety record and has been proposed as a pharmaceutical approach to inhibit aneurysm growth. However, it is unknown how the effect of doxycycline translates to the collagen homeostasis in human AAA disease. Therefore in **Chapter 3** we evaluated the effect of two weeks of doxycycline treatment on the activity of MMP and cysteine collagenases in patients scheduled for open repair. Results of this randomized clinical trial indicate that doxycycline improves the proteolytic balance through a dual effect on proteases and their inhibitors. Doxycycline treatment reduced MMP 8 and 9 expression and activation, and increased the levels of the protease inhibitors TIMP1 and cystatin C. Since the effects on MMP 8 and 9 expression were only observed at the protein level, we reasoned that this reduction reflects an effect on neutrophils. Indeed, evaluation of the neutrophil content in the aneurysmal wall revealed that the administration of doxycycline in patients with a large aneurysm over 14 days significantly reduced the number of infiltrating neutrophils in the tunica media and adventitia as compared with controls.

Neutrophils have long been considered part of an acute inflammatory response and their abundance in aneurysmal wall often been ignored. Recent studies, however, show that neutrophils may also participate in chronic inflammatory processes, and studies in mice show that neutrophils are critically involved in experimental AAA.[4,5] The presence of neutrophils in the aneurysmal wall, and therefore the effect of doxycycline, is not explained by the current concepts of inflammation in AAA. In **Chapter 4** we set out to explain the presence of neutrophils in human AAA, and contribute to the characterization of the inflammatory footprint of the disease. Results of this evaluation showed increased activation of general

pro-inflammatory transcription factors, accompanied by IL-6 and IL-8 hyperexpression, and exaggerated downstream cellular responses, particularly related to neutrophils.

As all these analyses were performed on surgical specimens collected during open repair, the data will therefore typically reflect the final stages of the disease process, and so do not allow distinction between primary and secondary (bystanding) processes. AAAs are epidemiologically associated with popliteal artery aneurysms (PAAs), suggesting that these two pathologies share common ground. In **Chapter 5** we carried out a systematic analysis of inflammatory and proteolytic pathways in AAA and PAA tissues to identify corresponding and discordant processes in these aneurysms. This, in turn, may provide clues on the processes that are crucial for aneurysmal growth. Findings show a remarkable overlap between the two diseases (AAA and PAA). Both are characterized by activation of the NF-κB and AP-1 pathways, IL-8 hyperexpression and neutrophil involvement.

In **Chapter 6** we showed that the profound suppression in neutrophil content after a short intervention with doxycycline is related to a selective suppression of inflammation of the vascular wall. This is reflected by inhibition of the AP-1 and C/EBP inflammatory pathways, and a strong reduction of the aortic wall neutrophil and cytotoxic T-cell content and activity. This study provides the rationale for the clinical evaluation of doxycycline as an anti-inflammatory drug in AAA disease.

Although impaired collagen metabolism is generally considered the hallmark of AAA progression, no clear collagen deficiency or defect has yet been identified in this disease. In **Chapter 7** we studied the collagen characteristics in AAA disease and showed that AAA is not characterized by a collagen deficiency but that the mechanical weakening relates to defects in the collagen micro-architecture, indicating a qualitative rather than a quantitative defect.

Future perspectives

Information on current treatments and the focus of future management

Traditionally, abdominal aneurysms were considered as a purely local mechanical dilatation of the aorta caused by atherosclerotic disease that ultimately led to rupture and death. In the last 50 years, much progress has been made in the surgical techniques of aortic repair. Surgical techniques have been developed based on the concept of mechanically excluding the aneurysm from the circulation, rather than targeting the processes driving aneurysm progression.

Aortic open repair and endovascular repair are now the mainstay of treatment. Unfortunately, both techniques remain associated with a significant mortality and morbidity. Therefore aneurysm repair (either open or endovascular) is only applied when risk of aneurysm rupture outweighs the risk of operation. Risk of rupture is largely dependent on the diameter of the aneurysm. Several large, randomized, prospective trials have evaluated the optimal size to treat abdominal aortic aneurysms.[6,7,8,9] The guidelines and conclusions from these studies

recommend intervention when the aneurysm reaches a transverse diameter of 5.5 cm. Currently, small aneurysms (i.e. diameter of 5.5 cm or less) are best approached with a wait-and-see policy. However, due to the aging population, the incidence of AAA is increasing, and screening of individuals which is proposed in a number of countries will detect many small asymptomatic AAAs. It would be beneficial to develop a medical management plan that would prevent the transformation of small aneurysms into large aneurysms, thereby decreasing the risk of rupture, and decreasing the number of therapeutic operations.

Furthermore, the presence of an abdominal aneurysm is associated with elevated rates of total mortality, cardiovascular disease mortality, and incident cardiovascular disease. These associations are independent of age, sex, other clinical cardiovascular disease, and extent of atherosclerosis.[10] This means that AAA disease is not only a local disease but also a symptom and a prognostic factor of significant cardiovascular systemic disease associated with morbidity and mortality. Therefore research must focus on the role of cardiovascular risk management in patients with AAA. It is expected that with this strategy, a decrease in the risk of mortality in patients with AAA could be achieved.

Current treatments (open and endovascular repair) are developed before understanding the pathology of abdominal aneurysms, and do not target the pathophysiology of aneurysm growth. Therefore we need a better understanding of the pathology to create opportunities for better treatments in patients with AAA.

Future perspectives on the pathophysiology of AAA

In this thesis we showed that human aneurysms are characterized by the destruction of the well-organized lamellar structure of the aorta. The proteins that are damaged include elastin and collagen, both highly stable proteins. Collagen is responsible for the mechanical stability of the healthy vessel wall.[11] We, among others, have implicated MMPs and cathepsins because of their ability to degrade collagen. We found abundant presence of multiple members of the MMP and cathepsin family in human AAA tissue. To find out if the identified MMPs and cathepsins are causatively involved in the pathophysiology of aneurysm disease or whether they are simply bystander proteins that accompany an inflammatory response, we need to further evaluate the role of the MMP's and cathepsin family members in animal models of AAA.

One of the true collagenases that is most abundantly present in our human aneurysm tissues is the neutrophil-derived MMP-8. Neutrophil depletion or intervention with neutrophil activation has been shown to prevent AAA formation in established animal models of the disease.[12,13] Although these observations support a role for neutrophils in AAA, they do not directly help to determine a effective and safe therapeutic strategy. Our findings show abundant expression of the chemokine IL-8, a strong neutrophil attractant. Therefore we hypothesize that blocking IL-8 signaling will reduce granulocyte recruitment to the vessel wall. IL-8 signalling is largely mediated by the CXCR2 receptor. Oral CXCR2 antagonists are currently under preclinical investigation. A next step would be to test these compounds

in established mouse AAA models.[14,15] Although MMP inhibitors failed during clinical evaluation,[16] a number cathepsin K and S inhibitors are in early clinical evaluation.[17,18] In light of this, we suggest to perform cathepsin inhibition in the mouse AAA model to evaluate the role of this class of proteases in aneurysm formation and progression.

Since inflammation is the primary driving force in the pathogenesis of AAA and major regulator in the production and activation of proteases, it would be of great interest to do additional studies to evaluate the role of inflammatory pathways in AAA disease. For example, the migration of leukocytes to sites of stress is regulated by chemokines and their receptors. In atherosclerosis, it has been shown that the monocyte chemoattractant protein-1 (MCP-1)/ chemokine receptor CCR2 pathway has a key role in leukocyte recruitment.[19,20,21] In addition, in our human aneurysm tissue specimens, we detected very high levels of MCP-1, suggesting an involvement for this chemokine in AAA. Indeed, in the mouse AAA model, transplantation of apolipoprotein E (ApoE)-deficient (hyperlipidemic) mice with bone marrow derived from CCR2/ApoE double-deficient mice largely prevented aneurysm formation.[22] This suggests that CCR2-mediated leukocyte recruitment has a major role in aneurysm formation. As an effective way to study the role of CCR2-mediated response in an animal aneurysm model, we could inhibit CCR2-mediated recruitment by applying gene transfer of a dominant-negative form of MCP-1 via *in vivo* electroporation in the skeletal muscle of the mice.[23]

Research is focusing on the role of the inflammatory and proteolytic factors in AAA animal models, providing better understanding of the pathology of AAA and defining therapeutic targets. However, there is an uncertainty as to how therapeutic targets that are set by animal models will translate to humans. AAA animal models, that are available, are based on artificial development of aneurysms, and their results should be interpreted with caution. The widely used angiotensin II/hypercholesterolemia models bear limited resemblance to human AAA, and appear to merely reflect dissection of the vessel wall.[24] As such, it holds limited information on the development of an aneurysm. Although the elastase and calcium chloride animal models are characterized by transmural inflammation and proteolytic imbalance, aneurysms in these models seldom rupture, questioning their relevance.[24] To identify effective therapeutic targets, we require to design an experimental model that represents the progression of an existing aneurysm, including rupture. Another important point of consideration is that most animal studies so far focus on the prevention of AAA development. However, the main focus of pharmaceutical strategies in man is on the prevention of AAA progression and, even more importantly, on prevention of rupture.

A number of observational studies have suggested that some antihypertensive drugs (ACE-inhibitors), non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics (doxycycline), and statins may reduce aneurysm growth rates. Unfortunately, the data has not yet be confirmed in larger studies. The observed sharp increase of cardiac disease, and the high incidence of cardiovascular death in AAA patients stresses the importance of cardiovascular risk management in this group. As such, the use of statins, antihypertensives and NSAIDs, together with lifestyle modification such as stopping smoking, should become standard

advice to patients with a small aortic aneurysm.[25] Further studies of these agents on AAA progression, and incident cardiac disease and death in AAA patients is warranted.

Perspectives on doxycycline use in patients with AAA

Doxycycline is a safe drug with immunomodulatory activities. It has been shown to inhibit the inflammation and proteases in the vessel wall and as such may reduce the growth of an AAA. Preclinical data clearly show that that doxycycline suppresses the formation of aneurysm in a number of animal models.[26] In this thesis, we showed that two weeks of doxycycline is safe and inhibits specific inflammatory and proteolytic pathways in large AAAs that required open surgical repair. A critical question is whether these observations will translate to small AAAs and whether long-term doxycycline treatment will result in inhibition of aneurysm growth. The efficacy of doxycycline remains to be established in a prospective, sufficiently powered clinical trial. Therefore we recently started a multicenter randomized trial, the Pharmaceutical Aneurysm Stabilisation Trial (PHAST), which evaluates the effects of doxycycline versus placebo on AAA growth in patients under surveillance for a small (3.5–5.0 cm) or patients with larger (over 5.5 cm) AAA who are unfit for, or refuse intervention. Outcome parameters of PHAST are aneurysm growth determined by ultrasound, possible side effects of doxycycline, need for elective aneurysm repair, rupture rate and mortality.

In this thesis we showed that the effect of doxycycline is specific on the C/EBP and AP-1 pathway and not on NF-kB pathway. This observation suggests that an intervention targeting NF-kB-regulated pathways may be complementary to doxycycline. Such a strategy could be considered when the effect of doxycycline on aneurysm growth is limited. *In vitro* studies show that statins, vitamin D receptor agonists and ACE inhibitors all reduce NF-kB activity.[27,28,29,30,31,32] Yet, it is unknown how these preclinical findings translate to the human situation. Future studies should focus on the effects of these compounds, and on the additive value of combination therapy of doxycycline, with the aforementioned drugs.

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Black sheep, 2007

Nederlandse samenvatting
Publications
Dankwoord
Curriculum Vitae

Nederlandse samenvatting

Een abdominale aneurysma (verwijding) van de grote lichaamsslagader komt veel voor bij ouderen. Terwijl kleine aneurysma's weinig klachten geven en een zeer geringe kans hebben om spontaan te scheuren (ruptureren) neemt de kans op scheuren bij grotere aneuysma's snel toe. Ondanks verbeteringen van de medische zorg overlijdt nog steeds 80% van de mensen bij wie een aneurysma ruptureerd. Het huidige beleid is er dan ook op gericht op het voorkomen van een ruptuur van het aneurysma. Hiervoor staan twee behandelingen ter beschikking: het operatief vervangen van de grote lichaamsslagader door een kunststof prothese of, indien mogelijk, het via de bloedbaan plaatsen van een prothese (stentprothese) binnen in het verwijde deel van de lichaamsslagader. Beide ingrepen zijn echter niet probleemloos: in ongeveer 3 % van de gevallen komt de patiënt te overlijden tijdens de ingreep terwijl het operatief vervangen van de lichaamsslagader een zware ingreep is waarvoor patiënten lang-during in het ziekenhuis moeten verblijven. Uit prospectief gerandomiseerd onderzoek is gebleken dat bij het plaatsen van een prothese via de bloedbaan (EVAR) er op de korte termijn de sterfte lager is dan die bij de conventionele techniek. Echter, op de langere termijn verdwijnt dit voordeel. EVAR kan dan vooral problemen geven door technische problemen van de stent. Momenteel gaan de operatieve behandeling gepaard met een relatief hoge mortaliteit en morbiditeit, er is een behoeft aan een medicamenteuze behandeling dat de goei van aneurysmata remt.

Het ontstaan van de abnormale verwijding van de grote lichaamsslagader en het vervolgens scheuren van het aneurysma wordt veelal geassocieerd met het verdwijnen van elastine, een belangrijk structureel eiwit van de vaatwand, en een chronische ontsteking. Onlangs is gesuggereerd dat het medicamenteus remmen van elastine afbraak operaties zou kunnen voorkomen. Echter, hoewel het verdwijnen van elastine uit de wand zeer karakteristiek is voor aneurysmavorming, blijkt uit microscopisch onderzoek dat het verdwijnen van elastine zeer vroeg optreedt in het proces van aneurysmavorming en dat ook in kleine aneurysma's vrijwel al het elastine reeds verdwenen is. De stevigheid van de wand van lichaamsslagader blijkt met name bepaald te worden door een ander structureel eiwit, collageen. De collageen afbraak in aneurysma's is verhoogd. Remmen van de verhoogde collageenafbraak is dan ook een aantrekkelijke optie om verdere groei van aneurysma's te voorkomen.

Afbraak van de structurele eiwitten in de wand van aneurysmata wordt met name toegeschreven aan eiwitten van de matrix metalloproteinase (MMP) familie, een grote familie met proteinases met een verschillende specificiteit. Voor de afbraak van collageen zijn primair de zgn collagenases (MMP 1, -8 en –13) verantwoordelijk, als deze eenmaal het collageen hebben aangetast dan zijn de gelastinases (MMP 2 en -9) in staat om het collageen verder af te breken. In dit proefschrift tonen we een sterk verhoogde expressie en activiteit van zowel collagenases als gelatinases in de aneurysmawand. Tevens wordt de ontstekings process in de aneurysma wand in kaart gebracht.

Van doxycycline, een veel gebruikt antibioticum, is bekend dat het de activiteit van MMP's en ontsteking remt. In diermodellen van aneurysmavorming is van doxycycline is aangetoond dat het de vorming van aneurysma's tegen gaat. Verder blijkt uit een een beperkt onderzoek bij de mens dat doxycycline de activiteit van MMP gelatinases in de aneurysmawand remt. Tot op heden is er geen goede verklaring voor het effect van de doxycycline op de aneurysma. Dit proefschrift laat het effect van verschillende doxycycline doseringen op de proteinases en ontsteking zien. De resultaten laten een remming zien van de proteinases en ontsteking en kan mogelijk de groei van een aneurysma belemmeren. De resultaten vormen de basis voor een groter prospectief multi-centre onderzoek naar het effect van langdurige behandeling met doxycycline op de groei van kleine aneurysma's.

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Dankwoord

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Hazem

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 24 oktober 1980 te Annaba. Het eindexamen VWO werd behaald in 2000 aan het Atlas College (R.O.S.) te Rijswijk. In datzelfde jaar werd begonnen met de studie geneeskunde aan de Rijksuniversiteit Leiden. Van 2002 tot 2004 werd parttime onderzoek verricht op de afdeling Nierziekte naar de identificatie van nieuwe complement activerende lectinen onder supervisie van Prof. dr. M.R Daha. Voorafgaand aan het behalen van het doctoraalexamen in 2006, werd gedurende 2 jaar fulltime onderzoek verricht op de afdeling Vaatchirurgie onder supervisie van Prof. dr. J.H. van Bockel, uiteindelijk heeft het onderzoek geleid tot dit proefschrift. In 2007 behaalde hij het bachelorexamen Wijsbegeerte en in 2008 het artsenexamen (cum laude) aan de Rijksuniversiteit Leiden. In 2010 begon hij aan de opleiding Neurochirurgie te Rotterdam onder leiding van Prof. dr. C.M.F. Dirven.

De afbeeldingen zoals hieronder weergegeven en verwerkt in het proefschrift, zijn geschilderd door de auteur gedurende de promotieperiode.


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Mirror city, 2008


The Green Man, 2009


Life in red, 2010


Happy birthday, 2009


Silent road, 2008


In a new world, 2008


Judgment, 2007


In the disco, 2007


Black sheep, 2007